

Glycoproteins of the Chromaffin Granule Membrane

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This thesis was composed by myself and all results
therein are based entirely on my own work.

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ABBREVIATIONS

ATP, adenosine 5' - triphosphate

ATPase, adenosine 5' - triphosphatase (E.C.3.6.1.3)

BSA, bovine serum albumin

Con A, concanavalin A

C₁₂E₈, octaethylene-glycol dodecyl ether

DBH, dopamine β -hydroxylase (E.C.1.14.17.1)

DCCD, NN' dicyclohexylcarbodiimide

DEAE, diethylaminoethyl

DTT, dithiothreitol

EDTA, ethylenediaminetetraacetic acid

F₁, soluble fraction of proton translocating ATPase

F₀, membrane-bound fraction of proton translocating ATPase

Fuc, fucose

Gal, galactose

GalNAc, N-acetylgalactosamine

Glc, glucose

GlcNAc, N-acetylglucosamine

Gp, glycoprotein

Hepes, N-2-hydroxyethylpiperazine-N'-2-ethane sulphane

HRP, horseradish peroxidase

SAM-HRP, horseradish peroxidase-sheep anti-mouse IgG conjugate

LCL, Lens culinaris lectin

Man, mannose

Mes, 4-morpholinoethanesulphonic acid

NeuAc, N-acetylneuraminic acid

NeuGc, N-glycolylneuramic acid

PMSF, phenylmethanesulphonyl fluoride

r.p.m., revolutions per minute

SA, sialic acid

SDS, sodium dodecyl sulphate

TEMED, NNN'N' - tetramethyl-1, 2-diaminoethane

TLCK, N α -p-tosyl-L-lysine chloromethyl ketone

TPCK, L-1-tosylamide 2-phenylethyl chloromethyl ketone

Tris, Tris-(hydroxymethyl) - aminomethane

$\Delta\psi$, electrical membrane potential

ΔpH , transmembrane pH gradient

WGA, wheat germ agglutinin

ABSTRACT

The membranes of chromaffin granules, the catecholamine-storing vesicles of the adrenal medulla, contain several major proteins, including glycoprotein J, glycoprotein K, cytochrome b-561, as well as membrane-bound forms of glycoprotein lll and dopamine β -hydroxylase (which also occur in the soluble phase). Glycoproteins J and K are uncharacterized proteins of unknown function. Dopamine β -hydroxylase, glycoprotein lll and glycoproteins J and K were solubilized from the granule membrane using the non-ionic detergent octaethylene-glycol dodecyl ether and purified by affinity chromatography on Lens culinaris lectin-agarose, followed by electrophoresis and electroelution from polyacrylamide gels. Purified glycoproteins were injected into guinea pigs and rabbits. The polyclonal antisera produced were used to characterize glycoproteins J and K and to compare their distribution with that of dopamine β -hydroxylase, glycoprotein lll and cytochrome b-561.

Glycoproteins J and K were shown to be specific components of the granule membrane; soluble forms were not detected in the matrix. These glycoproteins appear to span the membrane and presumably the carbohydrate portions are on the matrix (extracytoplasmic) side. Dopamine β -hydroxylase and glycoprotein lll were exposed on the matrix side and cytochrome b-561 is a transmembrane protein. Glycoprotein lll, which is confined to chromaffin granules in the adrenal medulla was also found in the pituitary.

Antibodies raised against glycoprotein J also reacted with glycoprotein K and vice versa; the relationship between glycoproteins J and K is discussed.

A monoclonal antibody was produced which recognized an epitope common to both soluble and membrane dopamine β -hydroxylase. Two novel proteins in the granule membrane were identified using monoclonal antibodies to the synaptic vesicle proteins p65 and synaptophysin.

CHAPTER ONE

INTRODUCTION

1.1 General Introduction

Chromaffin granules are membrane-bounded, catecholamine storing secretory vesicles, found in chromaffin cells in the adrenal medulla. They are similar in composition to the noradrenaline-containing synaptic vesicles in sympathetic nerves. Chromaffin cells were given their name because of the yellow-brown colour produced by the reaction of adrenaline with chromium salts. In the adrenal there are two types of chromaffin cell, secreting adrenaline and noradrenaline (Winkler and Carmichael, 1982; Phillips and Apps, 1979).

The contents of the granules are released by a process of exocytosis in which the chromaffin granule membrane fuses with the plasma membrane of the chromaffin cell. The major physiological stimulus for exocytosis in chromaffin cells is the release of acetylcholine from the terminals of the splanchnic nerve. In the case of bovine adrenal glands, acetylcholine acts on nicotinic receptors of chromaffin cells, depolarising the plasma membrane and stimulating a rise in free intracellular Ca^{2+} . Although the mechanism of the membrane fusion event of exocytosis is not understood in detail, it is known to require Mg - ATP, and to have a threshold for Ca^{2+} of about $1\mu\text{M}$ in leaky cells (Baker and Knight, 1984).

Chromaffin granules are 100 - 500 nm in diameter (mean, 280 nm in bovine adrenal glands) as determined by electron microscopy (Coupland, 1968). The granules appear to be roughly spherical, differences in shape

observed in the electron microscope probably being due to different fixation procedures and to variations in sectioning plane.

Adrenaline, noradrenaline and dopamine are found in the matrix (Winkler and Carmichael, 1982), although adrenaline and noradrenaline are not present together within the same granules (Coupland and Hopwood, 1966). The precursor for catecholamine biosynthesis in chromaffin cells is tyrosine which is hydroxylated by cytoplasmic tyrosine hydroxylase (E. C. 1. 14. 16. 2) to form dihydroxyphenylalanine (DOPA). This is the rate - limiting step in catecholamine biosynthesis. Cytosolic aromatic amino acid decarboxylase (E.C. 4. 1. 1. 28) then catalyses the decarboxylation of DOPA to form dopamine. Dopamine is transported across the granule membrane (see later). Dopamine β -hydroxylase (DBH; E. C. 1. 14. 17. 1) catalyses the synthesis of noradrenaline from dopamine inside the granule. In chromaffin cells containing adrenaline -storing granules noradrenaline is transported out of the granule into the cytoplasm where phenylethanolamine N-methyltransferase (PNMT; E. C. 2.1.1.28) transfers a methyl group from S-adenosylmethionine to form adrenaline, which is then transported back into the granules. However, chromaffin cells containing noradrenaline -storing granules lack the enzyme PNMT and therefore cannot synthesize adrenaline (reviewed by Winkler, 1976).

Crude granules can be prepared by differential

centrifugation of isotonic homogenates of adrenal medulla (Winkler and Carmichael, 1982). The crude granule fraction may be centrifuged through 1.6 M sucrose to yield a pellet of relatively pure granules (Smith and Winkler, 1967). Centrifugation through 1.8 M sucrose results in purer granules but the yield is smaller. Chromaffin granule membranes can be prepared from pure granules by hypotonic lysis. The membranes are pelleted by centrifugation and the supernatant (the lysate) contains the soluble contents from the matrix of the granules.

1.2 Chromaffin granule matrix (lysate)

The components of the granule matrix are listed in table 1.1. They include soluble proteins, nucleotides, catecholamines and other components, the pH of the matrix being about 5.5 (reviewed in Winkler and Carmichael, 1982).

1.3 Chromaffin granule membrane

The membrane is a lipid bilayer which behaves in freeze-fracturing like other membranes, being split along its hydrophobic part yielding a convex fracture face and a concave fracture face on the cytoplasmic half of the bimolecular leaflet (Kryvi et al, 1979). Membrane-intercalated particles can be seen on both faces but are more numerous on the concave one. These particles (9nm diameter) are probably membrane proteins. Under certain conditions the particles can move translationally and finally aggregate which is characteristic of a fluid mosaic (Singer and Nicolson, 1972). Further evidence for a bilayer arrangement of the granule

Table 1.1 Components present in the matrix of bovine chromaffin granules

<u>COMPONENT</u>	<u>APPARENT MOL. WT.</u>	<u>REFERENCE</u>
Dopamine β -hydroxylase (E.C.1.14.17.1)	290000	
Chromogranin A	72000	
Chromogranin B	100000	1
Chromogranin C	80000	2
Proteoglycan	86000-100000	3
Glycoprotein III	37000- 43000	4
Enkephalin precursors	(29000 (23000 (14000	5
Enkephalin convertases		
(1) Trypsin-like	20000	6
(2) Carboxypeptidase B - like	50000	7

CONCENTRATION (mM)

Catecholamines	580
Total nucleotides	180
ATP	130
Ca ²⁺	17
Mg ²⁺	5
Ascorbate	23

References: (1) Fischer-Colbrie and Frischenschlager (1985) (2) Fischer-Colbrie et al (1986); (3) Falkensammer et al (1985b); (4) Fisher-Colbrie et al (1984); (5) Udenfriend and Kilpatrick (1983); (6) Lindberg et al (1984); (7) Fricker and Snyder (1983). Other data from Winkler and Carmichael (1982).

Table 1.2 Proteins of the bovine chromaffin granule membrane that have been functionally identified.

<u>COMPONENT</u>	<u>APPARENT MOL. WT.</u>
Dopamine β -hydroxylase (E.C.1.14.17.1)	290000
Adenosine triphosphatase (E.C.3.6.1.3)	
ATPase I (5subunits)	400000
ATPase II	140000
Cytochrome b-561	27000
Catecholamine transporter	45000
Nucleotide transporter	?
Ca ²⁺ /Na ²⁺ antiporter	?
Actin	40000
α -actinin	96000
Phosphatidyl inositol kinase (E.C.2.7.1.67)	?
NADH: acceptor oxidoreductase (E.C.1.6.99.3)	?

Apparent molecular weights of the ATPases from Percy et al (1985). Other data from Winkler and Carmichael (1982).

membrane was obtained in experiments in which spin labels were introduced into various positions on the membrane fatty acids (Marsh et al., 1976). The membrane contains cholesterol, phospholipids and gangliosides (studies of composition are reviewed by Winkler and Carmichael, 1982). Those proteins of the chromaffin granule membrane which have been functionally (and in some cases structurally) identified are listed in table 1.2; however there are clearly many other polypeptides present.

Chromaffin granule membrane proteins are resolved by one-dimensional electrophoresis in sodium dodecyl sulphate (SDS) polyacrylamide gels into at least twenty bands (Winkler and Carmichael, 1982; Winkler and Westhead, 1980). Abbs and Phillips (1980) observed up to sixty bands but of these about twenty were present only in small amounts, and may well have been due to contaminants. Some proteins, which have different isoelectric points, comigrate on one-dimensional electrophoresis. These components are often resolved by two-dimensional electrophoresis (Apps et al., 1980; Gavine et al., 1984; Pryde and Phillips, 1986). Only a few proteins of the granule membrane have been functionally characterized. They include the proton-ATPase, catecholamine transporter, dopamine β -hydroxylase and cytochrome b-561 whose probable functions are shown in Figure 1.1

1.3.1 Cytochrome b-561

Cytochrome b-561 is an integral membrane protein and

Figure 1.1 Protons, catecholamines and electrons are transported into the matrix by proteins in the granule membrane. Proton-translocating ATPase hydrolyses ATP in the cytoplasm to form ADP and Pi, and the energy released is used to pump protons into the matrix. A membrane potential gradient and pH gradient are set up which drive the uptake of dopamine by the catecholamine transporter. Dopamine β -hydroxylase converts dopamine to noradrenaline, the electrons required being supplied by ascorbate, which is converted to semidehydroascorbate. Cytochrome b-561 may transfer electrons from the cytosol to semidehydroascorbate so regenerating the ascorbic acid.

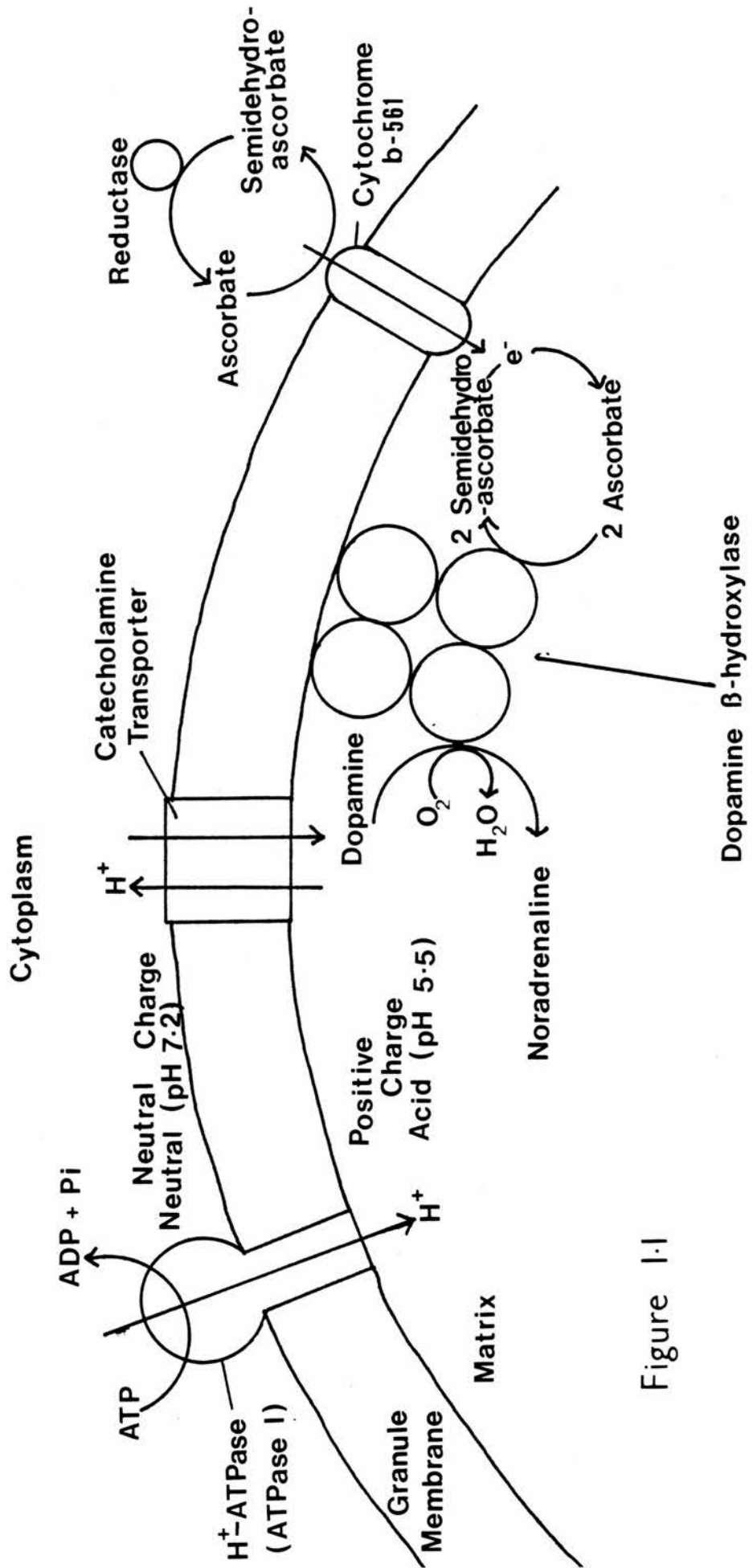


Figure 1.1

and is largely hydrophobic (Silsand and Flatmark, 1974). The prosthetic group is protohaem IX. It is thought to be the only haem-containing protein in chromaffin granule membranes and gives the membranes their pink colour (Terland et al., 1974). Cytochrome b-561 can be solubilized from the membrane with various detergents (Silsand and Flatmark, 1974; Apps et al., 1980; Duong and Fleming, 1982). Molecular weights of 22000-30000 have been reported (Apps et al., 1980; Duong and Fleming, 1982; Apps et al., 1984). Cytochrome b-561 is identical with chromomembrin B (Apps et al., 1980), a protein that was partially characterized by Hörtnagl et al., (1971).

Cytochrome b-561 does not contain carbohydrate (Huber et al., 1979). It shows complex redox behaviour, although the haem content of the cytochrome is 1 mol/mol protein (Apps et al., 1984). Proteolysis, immunological and chemical labelling studies have indicated that it is a transmembrane protein, with its major antigenic sites exposed on the cytoplasmic face of the granule (Duong and Fleming, 1984; Abbs and Phillips, 1980; Hunter et al.; 1982). Dopamine β -hydroxylase, which is located inside the granule, converts dopamine into noradrenaline and the electrons required for this reaction are donated by ascorbate, which is converted to semidehydroascorbate (Diliberto and Allen, 1981). As shown in figure 1.1, cytochrome b-561 is thought to transfer electrons across the membrane to semidehydroascorbate, thus renewing the supply of ascorbic acid (Srivastava et al., 1984; Njus et al., 1983).

Cytochrome b-561 also occurs in the membranes of the secretory vesicles found in the posterior pituitary gland (Duong et al., 1984). These vesicles do not contain dopamine β -hydroxylase. However, a similar mixed-function oxidase is present which appears to be involved in the α -amidation of the C-termini of oxytocin and vasopressin. Ascorbate appears to donate electrons to this mixed-function oxidase which cleaves a C-terminal glycine residue (Eipper et al., 1983). Cytochrome b-561 may have a similar transmembrane electron transport function in both pituitary granules and chromaffin granules.

1.3.2 Adenosine 5'-triphosphatases (ATPases; E.C.3.6.1.3)

The chromaffin granule membrane contains a proton-translocating ATPase which acidifies the granule matrix and generates a potential gradient across the granule membrane (positive inside). Accumulation of catecholamines in the granule is driven by both the pH gradient (ΔpH) and potential gradient ($\Delta\psi$) across the granule membrane (Johnson and Scarpa, 1976; Njus et al., 1981) and this is illustrated in figure 1.1. The catecholamine carrier will be discussed later. Transport of nucleotides into the matrix appears to be driven by $\Delta\psi$ alone (Weber and Winkler, 1981) but the carrier has not been characterized structurally,

An ATPase was isolated from granule membranes which is closely similar to F_1 -ATPase of mitochondria (Apps and Schatz, 1979). This ATPase probably represents contamination with the mitochondrial enzyme because most of the ATPase activity of the granules remains when the mitochondrial-type enzyme is removed with sodium bromide

(Cidon and Nelson, 1983; Cidon et al., 1983). A small ($M_r = 8000$), hydrophobic, DCCD-reactive protein is also present in granule membranes (Apps et al., 1983). This is similar in many ways to equivalent polypeptides that are components of the F_0 subunit of other proton-translocating ATPases (Sebald et al., 1979), and is thought to be a subunit of the proton-translocating ATPase (Sutton and Apps, 1981).

A novel ATPase (ATPase I) has been isolated from the chromaffin granule membrane (Percy et al., 1985). It behaves on gel filtration in the presence of detergent as a complex of apparent M_r 400000, is inhibited by DCCD and trialkyltin, and contains up to five subunits including the DCCD-reactive polypeptide (Percy et al., 1985). It has been shown unequivocally to function in proton translocation and hence in catecholamine transport. ATPase I is not glycosylated and can be separated from a second ATPase in the membrane (ATPase II).

ATPase II has an apparent M_r of 140000 and is strongly inhibited by vanadate (Apps et al., 1983; Percy et al., 1985). Its inhibitor sensitivity and substrate specificity differ from those of ATPase I. ATPase II does not translocate protons and its function is at present unknown (Percy et al., 1985).

1.3.3 Catecholamine transporter

Uptake of catecholamines into chromaffin granules depends on $\Delta\Psi$ and ΔpH (see fig. 1.1), generated by a proton-translocating ATPase (Johnson and Scarpa, 1976; Njus et al., 1981). The carrier transports adrenaline,

noradrenaline, 5-hydroxytryptamine (serotonin) and various catecholamine derivatives, such as tyramine and isoproterenol. Competition between these substrates indicates the presence of a single carrier (Phillips, 1974). Some progress has been made in identifying and isolating the catecholamine carrier, but the results are controversial (Gabizon et al., 1982; Scherman and Henry, 1983a). Most of the experimental evidence supports the hypothesis that one neutral catecholamine molecule is taken up in exchange for one proton (Scherman and Henry, 1981; Scherman and Henry 1983 b; Ramu et al., 1983). However, the kinetic experiments of Knoth et al. (1981) suggest that one molecule of protonated catecholamine is transported in exchange for two protons. In any event the transport is both electrogenic and pH-driven, and the mechanism proposed can account satisfactorily for the high catecholamine concentration gradient sustained across the granule membrane at equilibrium (Phillips and Apps, 1979).

1.4 Glycoproteins

Glycoproteins are found universally in living organisms. The plasma membrane of animal cells contains glycoproteins, for example erythrocyte glycophorin. Some enzymes and hormones are glycoproteins; examples are invertase and chorionic gonadotrophin. Serum glycoproteins include: IgG, thyroglobulin and prothrombin (Sharon and Lis, 1982). Glycoproteins are also found in the membranes and contents of intracellular organelles; examples being lysosomal hydrolases, HMG CoA reductase and ribophorins (Kornfeld and Kornfeld, 1985). Glycoproteins found in chromaffin granules will be discussed later.

The carbohydrate portion can be linked to protein either by an N-glycosidic bond to asparagine or by an O-glycosidic bond. There are four types of O-glycosidic linkage : (a) between N-acetylgalactosamine and serine or threonine, found in animal glycoproteins; (b) between xylose and serine, found in proteoglycans and human thyroglobulin; (c) between galactose and hydroxylysine in collagen; (d) between arabinose and hydroxyproline found in plant and algal glycoproteins (Sharon and Lis, 1982). Complex N-linked chains contain N-acetylglucosamine (GlcNAc), mannose (Man), galactose (Gal), fucose (Fuc) and sialic acid (SA). High mannose N-linked oligosaccharides contain GlcNAc and variable numbers of mannose residues. O-linked chains are comprised of N-acetylgalactosamine (GalNAc), galactose, fucose and sialic acid. The small number of sugar residues are linked together by different linkages and in various combinations to form a large number of oligosaccharide structures (Kornfeld and Kornfeld, 1985). The completeness of glycosylation can vary somewhat for a single glycoprotein species, giving rise to glycoprotein microheterogeneity. The polypeptide of an individual species is, of course invariant. Ovalbumin, for example, contains a mixture of molecules with nine different oligosaccharide structures attached to the same position of the polypeptide backbone (Sharon and Lis, 1982). Heterogeneous glycoproteins typically migrate as indistinct and poorly focussed bands on SDS polyacrylamide gels.

Isolation and purification of glycoproteins has

Table 1.3 Sources and specificities of lectins

LECTIN	SOURCE	SPECIFICITY	REF.
Concanavalin A (Con A)	Jack bean (<u>Canavalia</u> <u>ensiformis</u>)	α -D-Man > α -D-Glc α -D-GlcNAc	1,2
Lentil lectin (LCL)	<u>Lens culinaris</u>	α -D-Man > α -D-Glc > α -D-Glc	3
Horse gram lectin (DBA)	<u>Dolichos</u> <u>bifloros</u>	α -D-GalNAc	4
Ricinus commun- unis agglutinin (RCA)	Castor bean <u>Ricinus commu-</u> <u>nis</u>	β -D-Gal > α -D-Gal > α -D-GalNAc	5,6
Soybean agglutinin (SBA)	<u>Glycine max</u>	α -D-GalNAc > β -D-GalNAc	7
Gorse lectin (UEA)	<u>Ulex europaeius</u>	α -L-fucose	8
Wheat germ agglutinin (WGA)	<u>Triticum vulgar</u> <u>is</u>	β -D-GlcNAc	9
Helix pomatia lectin	Snail (<u>Helix</u> <u>pomatia</u>)	α -D-GalNAc	10
Pisum sativum	Garden pea (<u>Pisum sativum</u>)	α -D-Man, α -D-Glc	11
Peanut agglutinin(PNA)	<u>Arachis hypogaea</u>	β -D-Gal	12

References: (1) Goldstein and So (1965); (2) Goldstein et al. (1965); (3) Howard et al (1971); (4) Etzler and Kabat (1970); (5) Sharon and Lis (1972); (6) Takahashi et al. (1962); (7) Lis et al. (1970); (8) Matsumoto and Osawa (1970); (9) Burger and Goldberg (1967); (10) Hammarström et al. (1969); (11) Paulova et al. (1971); (12) Sharon and Lis (1982).

Gal, galactose; GalNAc, N-acetylgalactosamine;
Glc, glucose; GlcNAc, N-acetylglucosamine;
Man, mannose.

been facilitated by the use of lectins. These are non-immunoglobulin proteins from plants and animals that bind to specific monosaccharide residues (Briles and Kornfeld, 1978). A list of common lectins and their sugar specificities is given in table 1.3.

Glycoproteins which have the specific sugar residue in an exposed position in the oligosaccharide unit will bind noncovalently to either soluble or immobilized lectins. The complexes formed can be dissociated by adding an excess of the sugar for which the lectin is specific. This provides a method for separating glycoproteins that differ in their terminal sugar.

Glycoprotein carbohydrate chains have been found only on the extracellular surface of the plasma membrane (Sharon and Lis, 1982). To preserve the asymmetric arrangement of carbohydrates in the cell membrane, intracellular organelles that derive from or fuse with the membrane must have the carbohydrate of glycoproteins localized on the inner (extracytoplasmic) surface. Since chromaffin granules fuse with the membrane of the chromaffin cell it seems very unlikely that carbohydrates of glycoproteins are present on the cytoplasmic surface of the organelle membrane. Eagles et al (1975) found that ConA binds to isolated chromaffin granule membranes but not to intact granules suggesting that carbohydrates are on the inner (matrix) surface of the membrane. Isolated chromaffin granule membranes were treated with galactose oxidase and then with sodium boro [^3H] hydride and all the major glycoproteins

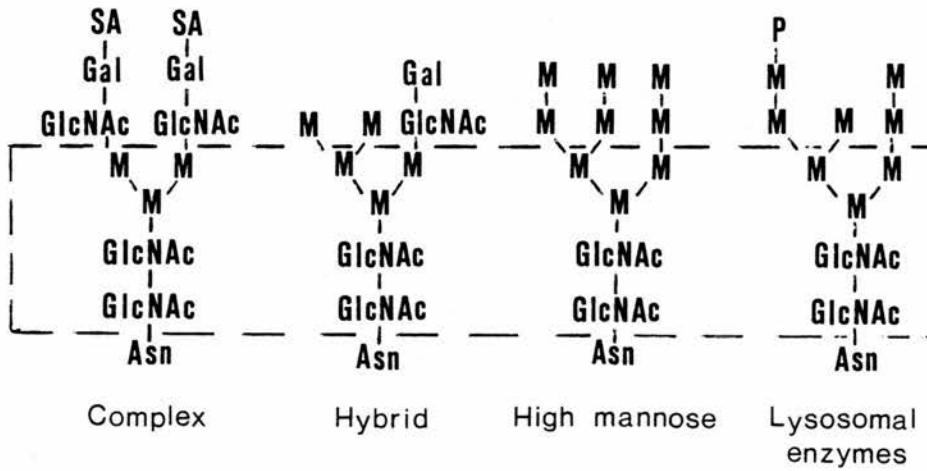


Figure 1.2 Structures of the major types of N-linked oligosaccharides. The boxed area encloses the pentasaccharide core common to all N-linked structures. Drawn from Kornfeld and Kornfeld (1985). Asn, asparagine; Gal, galactose; GlcNAc, N-acetylglucosamine; M, mannose; SA, sialic acid.

were labelled. There was no significant labelling of glycoproteins when intact granules were treated in this way (Huber et al., 1979; Abbs and Phillips, 1980). Although Meyer and Burger (1976) reported receptors for wheat germ agglutinin (WGA) on the cytoplasmic surface of intact granules, this work has not been confirmed.

1.5 N-linked oligosaccharides

Structures of the major types of N-linked oligosaccharides are shown in figure 1.2. Complex oligosaccharides may have more than two branches made up of different combinations of N-acetylglucosamine galactose and sialic acid residues (Kornfeld and Kornfeld, 1985).

1.5.1 Synthesis of the precursor oligosaccharide

The precursor of N-linked oligosaccharides is assembled on the lipid carrier dolichol phosphate (Dol-P). Sugars are added in a stepwise fashion with the first seven sugars (two GlcNAc and five Man residues) derived from the nucleotide sugars uridine diphosphate (UDP) - GlcNAc and guanosine diphosphate (GDP) - Man. The next seven sugars (four Man and three Glc residues) are derived from the lipid intermediates Dol P-Man and Dol P-Glc. Tunicamycin inhibits the transfer of GlcNAc from UDP-GlcNAc to Dol-P and this antibiotic may be used to study the function of the carbohydrate portion (Elbein, 1981). The structure of the precursor oligosaccharide is shown in figure 1.3. The precursor is transferred to a polypeptide chain in the lumen of the

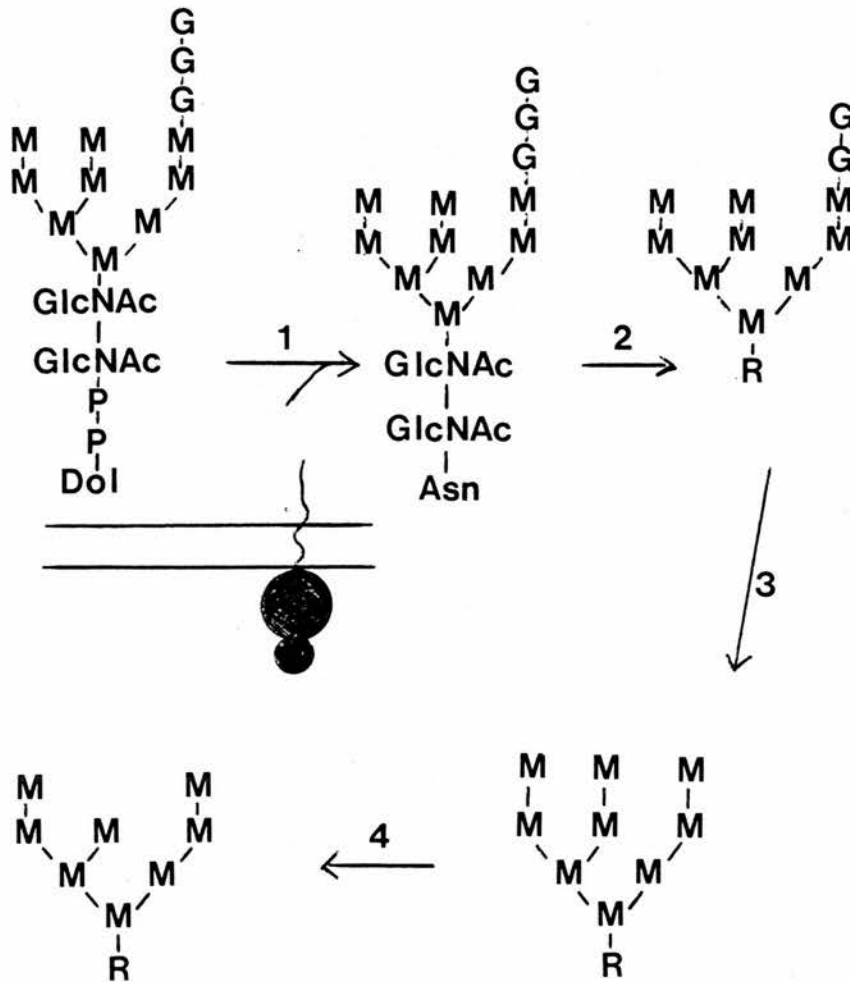


Figure 1.3 Assembly and processing of N-linked oligosaccharides in the rough endoplasmic reticulum.

The reactions are catalysed by the following enzymes:
 (1) oligosaccharyltransferase; (2) α-glucosidase I;
 (3) α-glucosidase II; (4) ER mannosidase.

Abbreviations: Asn, asparagine; Dol P P, dolichol diphosphate, G, glucose; GlcNAc, N-acetylglucosamine; M, mannose; R, (GlcNAc)₂ - asparagine (polypeptide chain).
 Drawn from Rothman (1985); Kornfeld and Kornfeld (1985).

rough endoplasmic reticulum (RER) but it is not known where the precursor is assembled (Kornfeld and Kornfeld, 1985; Snider et al., 1984)

1.5.2 Processing in the rough endoplasmic reticulum

The precursor oligosaccharide unit is usually transferred from dolichol diphosphate to an asparagine in a nascent polypeptide during its vectorial transport across the membrane of the RER (Kornfeld and Kornfeld, 1985). Glycosylated asparagine residues almost always occur in the sequence Asn-X-Ser/Thr where X can be any amino acid except possibly proline and aspartic acid (Marshall, 1972). Nevertheless, not all Asn-X-Ser/Thr sequences in suitable proteins are glycosylated, so the protein structure must play some role in determining glycosylation sites. The sequence of processing steps in the RER is illustrated in figure 1.3. Three glucose residues and a mannose residue are removed from all N-linked glycoproteins by enzymes located in the membrane of the RER. This can occur either cotranslationally or posttranslationally (Kornfeld and Kornfeld, 1985, Schwarz and Datema, 1984). Further mannose residues are removed from the ER glycoproteins HMG CoA reductase and ribophorins. With the exception of ER membrane glycoproteins, the newly synthesized glycoproteins are next transported to the cis Golgi cisternae. Translocation is probably mediated by vesicles which bud from the RER and then fuse with the Golgi membrane. Removal of the glucose residues may be necessary for movement from the RER to the Golgi or alternatively, for the glycoprotein to

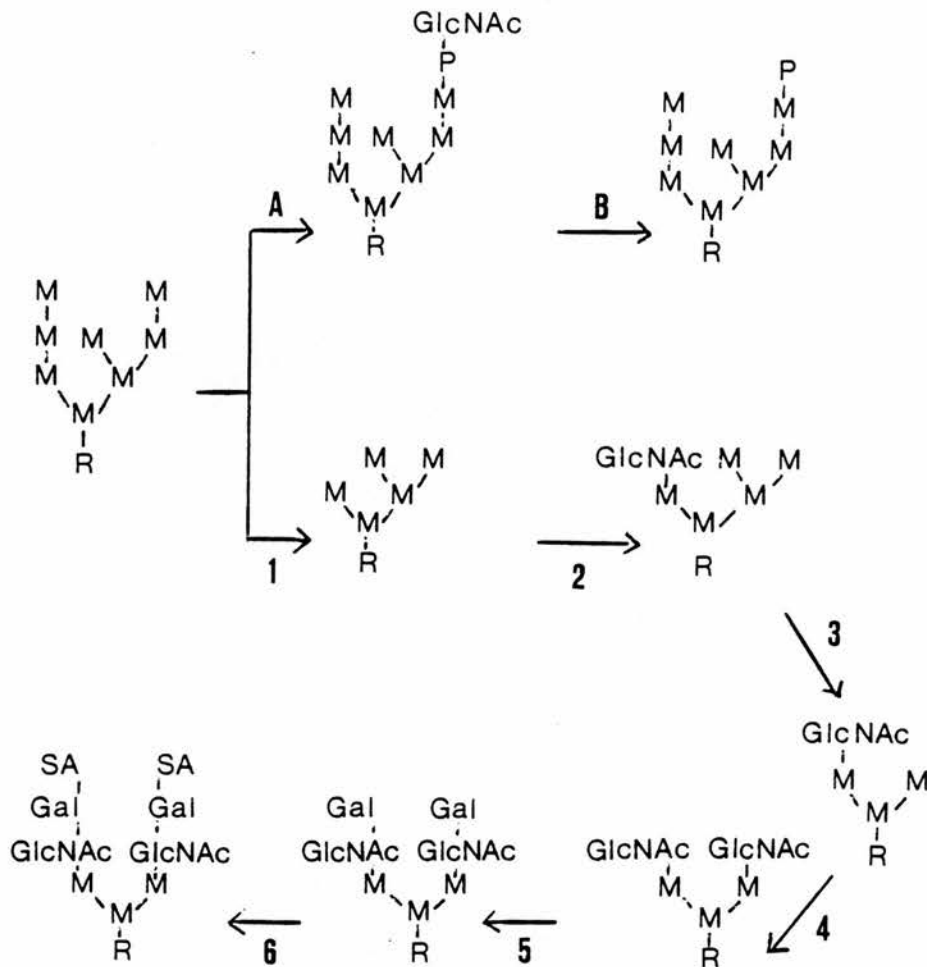


Figure 1:4 Assembly and processing of N-linked oligosaccharides on plasma membrane, secretory and lysosomal proteins, in the Golgi apparatus. Steps 1-6, formation of a complex type chain. The enzymes catalysing each step are: (1) mannosidase I; (2) Glc NAc - transferase I; (3) mannosidase II; (4) Glc NAc - transferase II; (5) galactosyltransferase; (6) sialyltransferase. The product is a biantennary (two branched) chain. Processing by other Glc NAc - transferases yields chains with additional branches, or chains with branches in different positions Steps A and B phosphorylation of lysosomal enzymes. (A) Glc NAc - phosphotransferase attaches a Glc NAc-phosphate group to one or two mannose residues. Any of five mannose residues may be phosphorylated (B) Glc NAc - phosphoglycosidase removes Glc NAc.

Abbreviations: Gal, galactose; GlcNAc, N-acetylglucosamine; M, mannose; R, (GlcNAc)₂ - asparagine (polypeptide chain); SA, sialic acid.

Drawn from Dunphy and Rothman (1985).

mature to a correct functional conformation.

1.5.3 Processing in the Golgi apparatus

The many different types of glycoprotein arriving at the cis face of the Golgi stack have identical oligosaccharide chains (Kornfeld and Kornfeld, 1985). Inside the stack the oligosaccharides are processed and glycoproteins are sorted, by an unknown mechanism, according to destination. The Golgi apparatus is divided into at least three compartments (cis, medial and trans), each compartment containing the enzymes needed for particular reactions in the processing of oligosaccharides (Rothman, 1985). The reason for compartmentalization is not known but it is likely to be important for sorting glycoproteins (Dunphy and Rothman, 1985).

The sequence of reactions that occur during the processing of N-linked oligosaccharides are shown in figure 1.4. Most lysosomal enzymes are phosphorylated on mannose residues and this is important for targeting the enzymes to lysosomes. Secretory and plasma membrane proteins typically undergo much more extensive processing to form high mannose or complex oligosaccharides (Kornfeld and Kornfeld, 1985).

Neutra and Leblond (1966) showed that sugar residues are added to protein in the Golgi apparatus. The location of the N-linked processing reactions in the cis, medial and trans compartments of the Golgi apparatus is summarized in figure 1.5. Membranes from Chinese hamster ovary cells were fractionated on sucrose gradients into dense membranes containing mannosidase I, GlcNAc-transferase I,

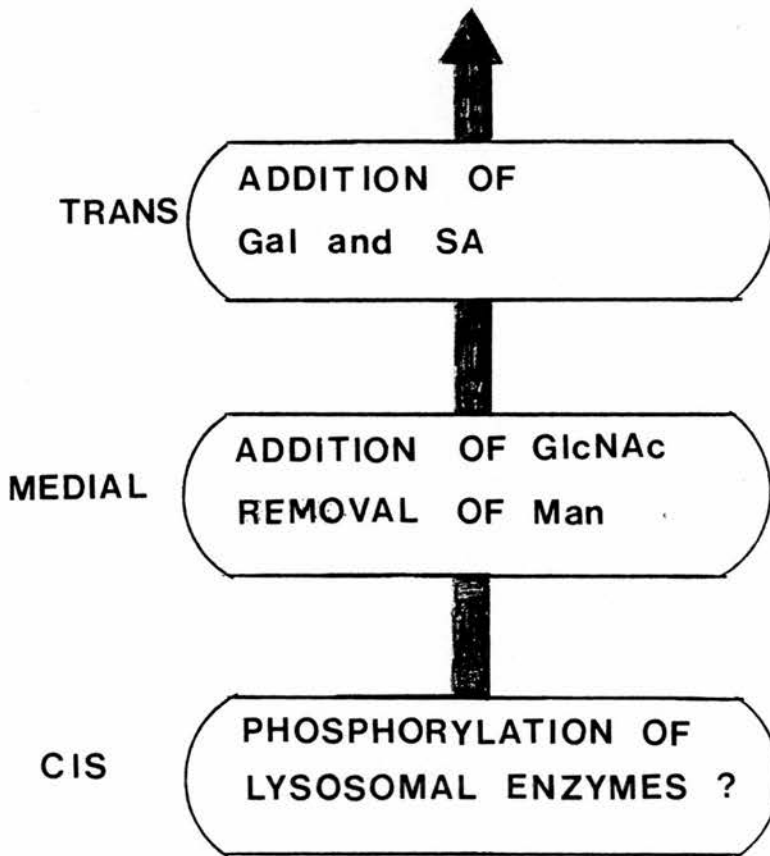


Figure 1.5 Summary of N-linked oligosaccharide processing reactions in the cis, medial and trans compartments of the Golgi apparatus.
Drawn from Dunphy and Rothman (1985).

mannosidase II, and GlcNAc-transferase II, and light membranes containing the enzymes that add galactose and sialic acid (Dunphy and Rothman, 1983). The two enzymes that phosphorylate mannose residues on lysosomal enzymes were found in an even denser population of membranes than those containing the GlcNAc-transferases (Goldberg and Kornfeld, 1983).

Some enzymes were found in compartments of the intact Golgi apparatus by electron microscopy. Electron-dense colloidal gold particles coated with S.aureus protein A were used to locate the binding sites of antibodies to galactosyltransferase within the trans Golgi (Roth and Berger, 1982). Dunphy et al (1985) using monoclonal antibodies, found that GlcNAc - transferase I is located in the medial cisternae; GlcNAc - phosphotransferase and phosphoglycosidase which phosphorylate lysosomal enzymes have not been located but they may occur in the cis Golgi. The galactose - specific lectin, ricin binds to oligosaccharides in the trans cisternae showing that galactose residues are added in this region.

There are two models to explain how proteins move through the Golgi. In the cisternal-progression model, a cisterna is formed at the cis face from vesicles derived from the ER. Each cisterna moves through the Golgi as an intact unit and when it reaches the trans face it gives rise to secretory vesicles (Farquhar and Palade, 1981). Because enzymes that process N-linked oligosaccharides are located in specific compartments, cisternae would have to acquire enzymes and then release them as they pass through

the stack if the cisternal-progression model is correct (Dunphy and Rothman, 1985).

Transfer from one cisterna to the next is more likely to occur by vesicles that bud from one cisternal compartment and fuse with the next. Vesicles budding from cisternae have been observed in the electron microscope. Cell fusion studies show that proteins can be transported from one cisterna to the next in an irreversible, vectorial process. Complex oligosaccharides are not formed in mutant Chinese hamster ovary cells which lack GlcNAc - transferase. Mutant cells infected with vesicular stomatitis virus (VSV) were fused with uninfected wild type cells. A proportion of the G protein produced by the fused cells was resistant to endoglycosidase H and so the oligosaccharide portion had been converted into the complex form. The glycoprotein must have passed from mutant to wild type Golgi cisternae for glycosylation to be completed (Rothman et al., 1984 a). Also, G protein which has received peripheral GlcNAc in one cell is efficiently transferred to the Golgi complex of another cell to receive galactose (Rothman et al., 1984 b)

Monensin (H^+ , K^+ and Na^+ ionophore) is used to study the passage of secretory proteins through the Golgi. It arrests intracellular transport of newly synthesized secretory proteins, proteoglycans and plasma membrane glycoproteins in the Golgi apparatus (Tartakoff, 1983). The effect of monensin on glycosylation depends on the particular glycoprotein and the cell in which glycosylation takes place. In hepatoma cells infected with VSV treated

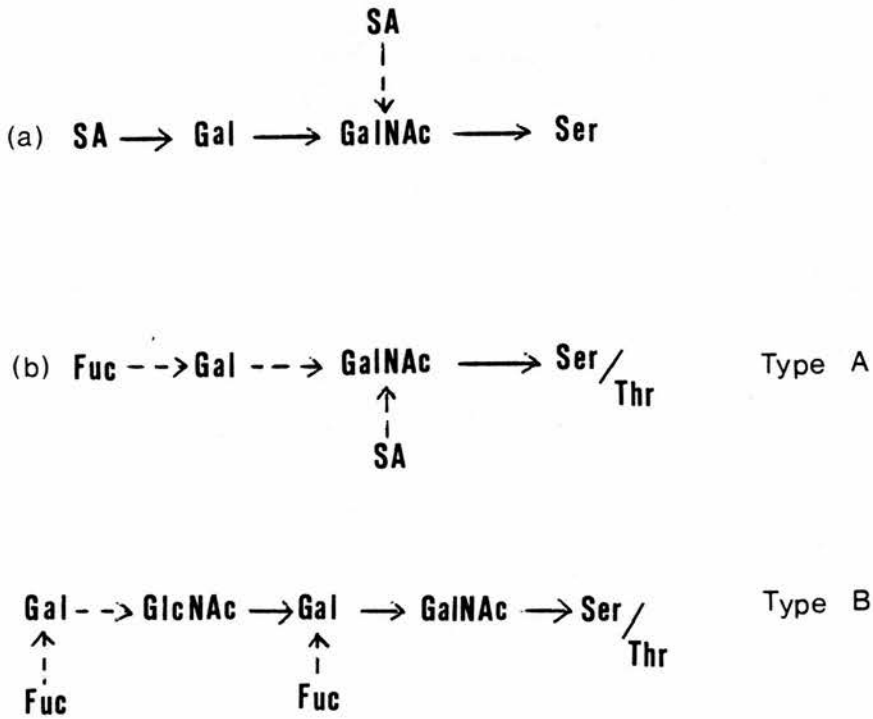


Figure 1.6 O-linked oligosaccharide chains of (a) fetuin (Spiro and Bhoyroo, 1974) and (b) canine submaxillary mucin (Lombart and Winzler, 1974). \dashrightarrow indicates residues which may be present. Abbreviations: Gal, galactose; GalNAc, N-acetylgalactosamine; SA, sialic acid; Ser, serine; Thr, threonine.

with monensin, newly synthesized transferrin was endoglycosidase H-sensitive but VSV G protein became resistant to the enzyme, even though both proteins were found in the same Golgi compartment (Tartakoff, 1983). Therefore, monensin treatment is not a reliable method for locating specific glycosylation reactions within the Golgi.

1.6 O-linked oligosaccharide chains.

These chains are commonly linked to the hydroxyl groups of serine or threonine by an N-acetylgalactosamine (GalNAc) residue. The structures of these oligosaccharide chains vary from disaccharides composed of GalNAc and sialic acid to large oligosaccharides of twenty sugar residues containing GalNAc, galactose, GlcNAc, fucose and sialic acid (Hanover and Lennarz, 1981). Some glycoproteins contain both N - and O - linked chains, e.g. glycophorin and human chorionic gonadotrophin. Typical O-linked oligosaccharides are shown in figure 1.6.

There is less information about the synthesis of O-linked oligosaccharides than that of N-linked chains. The first step involves the transfer of GalNAc from UDP-GalNAc to a serine or threonine residue by a UDP-GalNAc: polypeptide transferase. This does not require oligosaccharide preassembly nor lipid intermediates (Hanover and Lennarz, 1981). There is evidence that O-glycosylation occurs in the Golgi apparatus. The four glycoproteins found in the envelope of herpes simplex virus type 1 (HSV-1) contain N- and O-linked chains (Johnson and Spear, 1983). Monensin treatment of cells infected with HSV-1 blocks O-glycosylation of HSV-1

glycoproteins. Because monensin blocks intracellular transport within the Golgi (Tartakoff, 1983), O-glycosylation may occur during the passage of proteins through the Golgi (Johnson and Spear, 1983). Mucins synthesized by intestinal goblet cells contain O-linked oligosaccharide chains with terminal GalNAc residues (Roth, 1984).

Helix pomatia lectin (specific for GalNAc) complexed to gold particles was used to locate the site of attachment of GalNAc residues to protein in goblet cells.

Electron micrographs showed that most labelling was found in the cis Golgi, essentially none in the medial Golgi and some in the trans Golgi. This evidence indicates that core GalNAc residues are transferred to the polypeptide in the cis Golgi and terminal GalNAc residues are added in the trans Golgi (Roth, 1984). It is not clear whether the three regions defined in this study correspond precisely to the cis, medial and trans compartments that process N-linked chains (Dunphy and Rothman, 1985).

1.7 Function of the carbohydrate portion of glycoproteins

Glycosylation is a costly process for the organism in terms of energy and materials, suggesting that the oligosaccharides have important biological functions. Carbohydrate is not required for secretion of procollagen, ovalbumin and transferrin but it is necessary for the release of invertase and acid phosphatase from yeast. The activities of invertase and fibronectin are not affected by deglycosylation (Olden et al., 1982). However,

removal of carbohydrate from human chorionic gonadotrophin reduced the activity of the hormone without affecting binding to its receptor (Kalyan and Bahl, 1982). Many proteins including invertase, ribonuclease B and fibronectin are cleaved by proteases after removal of the oligosaccharides. Enhanced proteolysis of non-glycosylated secretory proteins may result from exposure to lysosomal hydrolases during secretion (Olden et al., 1982). Therefore, the role of the carbohydrate portion may be to protect the polypeptide chain from proteolysis. Surface sugars are involved in cell-cell interaction. Influenza virus binds to sialic acid residues on the surface of erythrocytes and treatment of erythrocytes with neuraminidase, which removes sialic acid residues, prevents binding (Sharon, 1980). Bacteria possess surface lectins which bind to carbohydrates on the surface of host cells and this step is essential for infection (Ofek et al., 1978). Glycoproteins are involved in the interaction of the sea urchin sperm with the egg during fertilization (Vacquier, 1986). Bindin, a protein which coats the acrosomal process of the sperm binds to glycoprotein receptors on the vitelline layer of the egg. Tunicamycin prevents N-linked glycosylation by inhibiting the transfer of GlcNAc to dolichol phosphate (Elbein, 1981). Tunicamycin inhibits the development of amphibian and mammalian embryos so glycoproteins may be involved in embryonic differentiation (Olden et al., 1982).

Mannose phosphate residues on lysosomal hydrolases are recognition markers that allow binding to the

mannose 6-phosphate receptor in the Golgi and subsequent translocation to lysosomes (Kornfeld and Kornfeld, 1985). Uptake of lysosomal hydrolases into human fibroblasts depends on the interaction of mannose phosphate residues with a mannose phosphate receptor on the cell surface (Gonzalez-Noriega et al. 1980; Fischer et al., 1980). Fibroblasts from patients with I-cell disease (a lysosomal enzyme storage disease) are deficient in GlcNAc-phosphotransferase which catalyses the first step in the phosphorylation of mannose residues in the Golgi. Newly synthesized enzymes lacking the mannose phosphate marker are secreted from cells instead of being translocated to lysosomes (Kornfeld and Kornfeld, 1985).

Secretory , plasma membrane and organelle glycoproteins are sorted in the Golgi. Mannose phosphate residues are markers for lysosomal enzymes but do sugar residues indicate the final destination of other glycoproteins? Each glycoprotein could have a certain carbohydrate structure which is recognized by a receptor. However, oligosaccharide structures can vary within a single glycoprotein species (microheterogeneity) and a receptor might not recognize all the structures. The polypeptide chain may contain most of the information about the destination of heterogeneous glycoproteins. If sugar residues are markers for the destination of glycoproteins then unique carbohydrate structures should be found in different regions of the cell. Although the carbohydrate structures of only the more abundant secretory and plasma membrane proteins are known, lysosomal enzymes

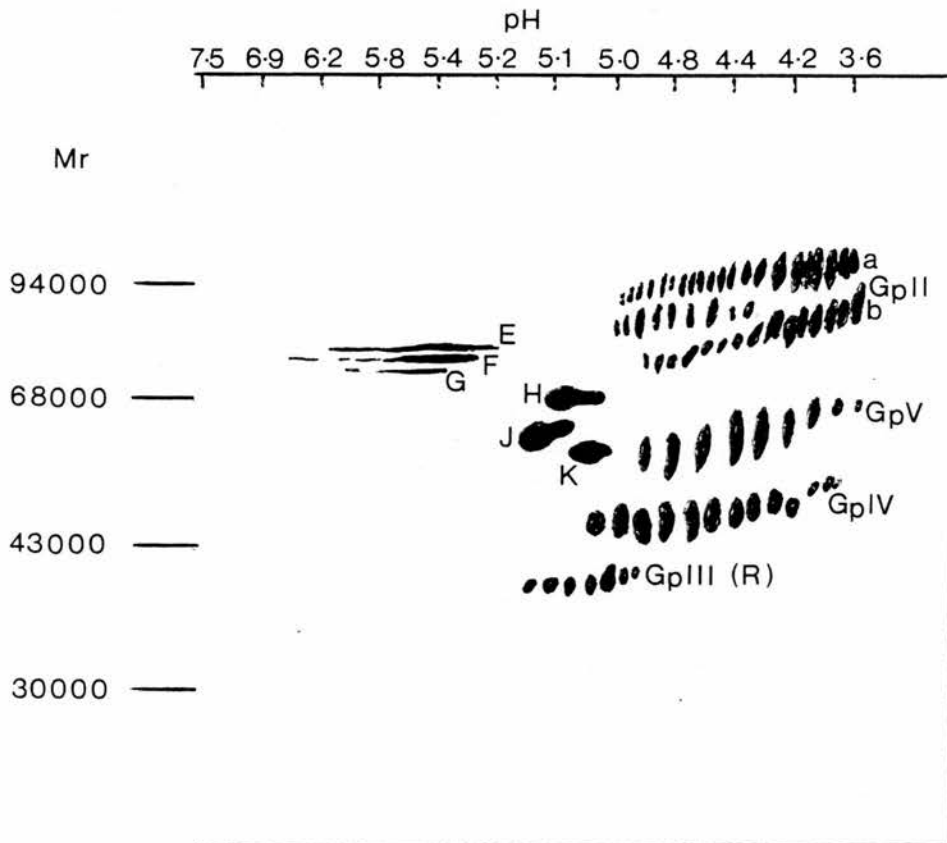


Figure 1.7 Schematic drawing of the major concanavalin A-binding glycoproteins of the chromaffin granule membrane. Labelling follows that used by Huber et al. (1979), Gavine et al. (1984) and Pryde and Phillips (1986). E, F and G are the components of dopamine β -hydroxylase, as revealed by antibody binding.

generally contain the mannose 6-phosphate marker.

Sugar residues on plasma glycoproteins are important for recognition of these compounds and their clearance from the plasma. Removal of sialic acid residues from ceruloplasmin exposes galactose residues that are recognized by receptors on liver hepatocytes, and asialoceruloplasmin is taken up into the cells (Ashwell and Morell , 1974).

1.8 Glycoproteins found in chromaffin granules

The chromaffin granule membrane contains approximately fifteen glycoproteins; the most abundant of which is dopamine β -hydroxylase (Gavine et al., 1984). Glycoproteins stain poorly with Coomassie Blue, so in early studies gels of membrane constituents were stained with periodic acid-Schiff reagent. This is relatively insensitive, but stains carbohydrates, particularly those containing sialic acid. Five glycoproteins were revealed, including dopamine β -hydroxylase which was then called glycoprotein I (Huber et al., 1979). Lectins have been used to identify and purify chromaffin granule glycoproteins (Cahill and Morris, 1979; Gavine et al., 1984; Apps et al., 1985; Pryde and Phillips, 1986). Most of the membrane glycoproteins bind Con A (Fig. 1.7). However, the low affinity of glycoprotein III for Con A makes it difficult to detect on Con A overlays of chromaffin granule membranes (Pryde and Phillips, 1986). Both glycoprotein II and glycoprotein III have a high affinity for WGA. The major matrix glycoproteins together with their lectin

Table 1.4 Lectin - binding properties of the major matrix glycoproteins of chromaffin granules

	ConA	ConA/ NEUR	PNA	PNA/ NEUR	PSA	PSA/ NEUR	WGA	WGA/ NEUR
Chromogranins A	-	-	-	+	-	-	-	-
Chromogranins B	+	+	-	+	+	+	+	+
Chromogranins C	ND	ND	ND	ND	ND	ND	ND	ND
Dopamine β -hydroxylase	+	+	-	-	+	+	+	+
Glycoprotein III	+	+	-	-	ND	ND	+	+
Proteoglycan	ND	ND	ND	ND	ND	ND	ND	ND

Data from Apps et al. (1985)

NEUR, neuraminidase treated; Con A, conanavalin A;
PSA, Pisum sativum agglutinin; WGA, wheat germ
agglutinin.

Sugar specificities of the lectins are given in table
1.3.

ND, not determined

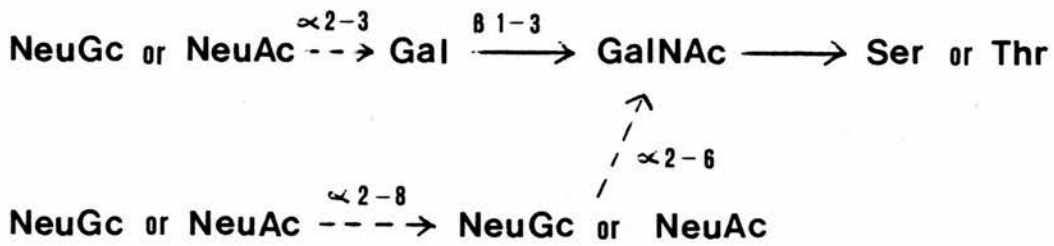


Figure 1.8 Structure of chromogranin A oligosaccharides
Dashed arrows indicate residues which are not present in all oligosaccharides. Data were obtained from Kiang W-L et al (1982).

Gal, galactose; GalNAc, N-acetylgalactosamine; NeuGc, N-glycolylneuraminic acid; NeuAc, N-acetylneuraminic acid; Ser, serine, Thr, threonine

binding properties are listed in table 1.4.

1.8.1 Chromogranins and the proteoglycan

The soluble proteins of chromaffin granules have been collectively called chromogranins (Blaschko et al., 1967). Chromogranin A (Mr 72000, pI 4.7 - 4.9) is their major component, comprising 40% of the total soluble protein (Winkler, 1976). Antiserum directed against chromogranin A reacts with a family of acidic proteins ranging in apparent Mr from 70000 to 17000 (Kilpatrick et al., 1983; Fischer-Colbrie and Frischenschlager, 1985). Chromaffin granules presumably contain an endogenous protease which cleaves chromogranin A to form these smaller proteins (Winkler, 1976). Although chromogranin A has been claimed to be an integral membrane protein (Settleman et al., 1985), this seems unlikely in view of its behaviour on phase-partition in Triton X-114 (Pryde and Phillips, 1986). Chromogranin A is almost completely removed from membranes by washing with sodium carbonate (Pryde and Phillips, 1986), indicating that the chromogranin A associated with the membrane is a contaminant. Chromogranin A has been found in several endocrine tissues (O'Connor et al., 1983; Cohn et al., 1982) and in brain (Somogyi et al., 1984). There are two other families of acidic glycoproteins found in chromaffin granules and brain, which have been termed secretogranins I and II by Rosa et al. (1985 a,b); these differ from chromogranin A with regard to their immunological properties and the peptides produced after cleavage with trypsin. Secretogranin II is

immunologically identical with chromogranin C (see below). Despite the widespread distribution of chromogranin A, its function is unknown. Chromogranin A binds Ca^{2+} (Reiffen and Gratzl, 1986) so it may help to regulate the free Ca^{2+} concentration within chromaffin granules.

The carbohydrate portion of chromogranin A, which makes up 5.4% of the glycoprotein, consists of tetrasaccharides or trisaccharides (see fig. 1.8) linked to serine or threonine by O-glycosidic bonds (Kiang et al., 1982). Chromogranins A are not decorated by Con A, Pisum sativum agglutinin (PSA) or peanut agglutinin (PNA), following transfer to nitrocellulose sheets (Apps et al., 1985). Galactose-specific PNA binds to chromogranin A after removal of terminal sialic acid residues by neuraminidase (table 1.4). These results are consistent with the structure of the oligosaccharide portion shown in figure 1.8.

Chromogranins B are another family of acidic proteins ranging in apparent Mr from 100000 to 24000, focussing at pI 5.2. The chromogranin B family are immunologically distinct from chromogranins A (Fisher-Colbrie and Frischenschlager, 1985). Unlike chromogranin A, chromogranins B bind both Con A and PSA (see table 1.4), which are specific for mannose (Apps et al., 1985). Higher molecular weight members of the family also bind PNA after neuraminidase treatment, so probably contain N-linked chains consisting of mannose residues, and O-linked chains of galactose and sialic acid.

Two precursors of chromogranin A are synthesized in reticulocyte lysate and wheat germ cell free systems (Kilpatrick et al., 1983; Falkensammer et al., 1985 a). Only one precursor of chromogranin B is produced when mRNA from the adrenal medulla is translated in a wheat germ cell free system. In the presence of microsomes the two chromogranin A precursors are converted into a single protein, apparently by the removal of different signal peptides (Falkensammer et al., 1985 a). Endogenous chromogranin A and chromogranin B are larger and more acidic than the precursors translated in the presence of microsomes and O-glycosylation in the Golgi probably accounts for the increase in Mr. Mature chromogranin B focusses at pI 5.2 but the precursor has a pI of 5.7. Isolated chromaffin cells were pulse labelled with [³⁵S] sulphate and chromogranin B was labelled more than chromogranin A (Falkensammer et al., 1985 b). The presence of sulphate residues in the mature form of chromogranin B may explain why it is more acidic than its precursor.

A third family of acidic proteins, chromogranins C are present in the matrix (Fischer-Colbrie et al., 1986). These proteins react with an antiserum raised against secretogranin II, a protein described by Rosa and Zanini (1983) in pituitary. Chromogranins C are immunologically distinct from chromogranins A and B, the largest component has a Mr of 86000 and a pI of 5.0. In a cell free system only one protein is synthesized which can be precipitated with anti-secretogranin serum

(Fischer-Colbrie et al., 1986).

A proteoglycan is found in the matrix of chromaffin granules (Falkensammer et al., 1985 b). This is a heterogeneous sulphated component (Mr 86000-100000; pI 4.3 - 5.0), susceptible to digestion with chondroitinase ABC.

1.8.2 Dopamine β -hydroxylase (DBH; E.C. 1. 14. 17. 1)

Dopamine β -hydroxylase was first isolated from bovine adrenal glands in 1960 (Levin et al., 1960). It is present as soluble and membrane-bound forms in chromaffin granules; the soluble form comprising 4% of the total soluble protein and the membrane-bound form 25% of the total membrane protein (Winkler, 1976). The native form of DBH is a tetramer of Mr 290000 (Wallace et al., 1973). In SDS two dimers of apparent Mr 150000 are formed and in the presence of 2-mercaptoethanol these further dissociate into monomers of Mr approximately 75000 (Winkler, 1976). DBH has a broad pI of 5.8 - 6.7 (Gavine et al., 1984).

DBH is a copper-containing enzyme that catalyses the conversion of dopamine to noradrenaline (Friedman and Kaufman, 1965) and this function of DBH is illustrated in figure 1.1. The active site of membrane DBH is on the interior surface of the granule membrane and that of soluble DBH in the granule matrix. This was pointed out by Kirshner (1962), who showed that dopamine had to be transported into the interior of the granule to be hydroxylated and this was later confirmed by the latency studies of Laduron (1975).

DBH is a glycoprotein, containing 4% carbohydrate. It is stained by periodic-acid Schiff reagent

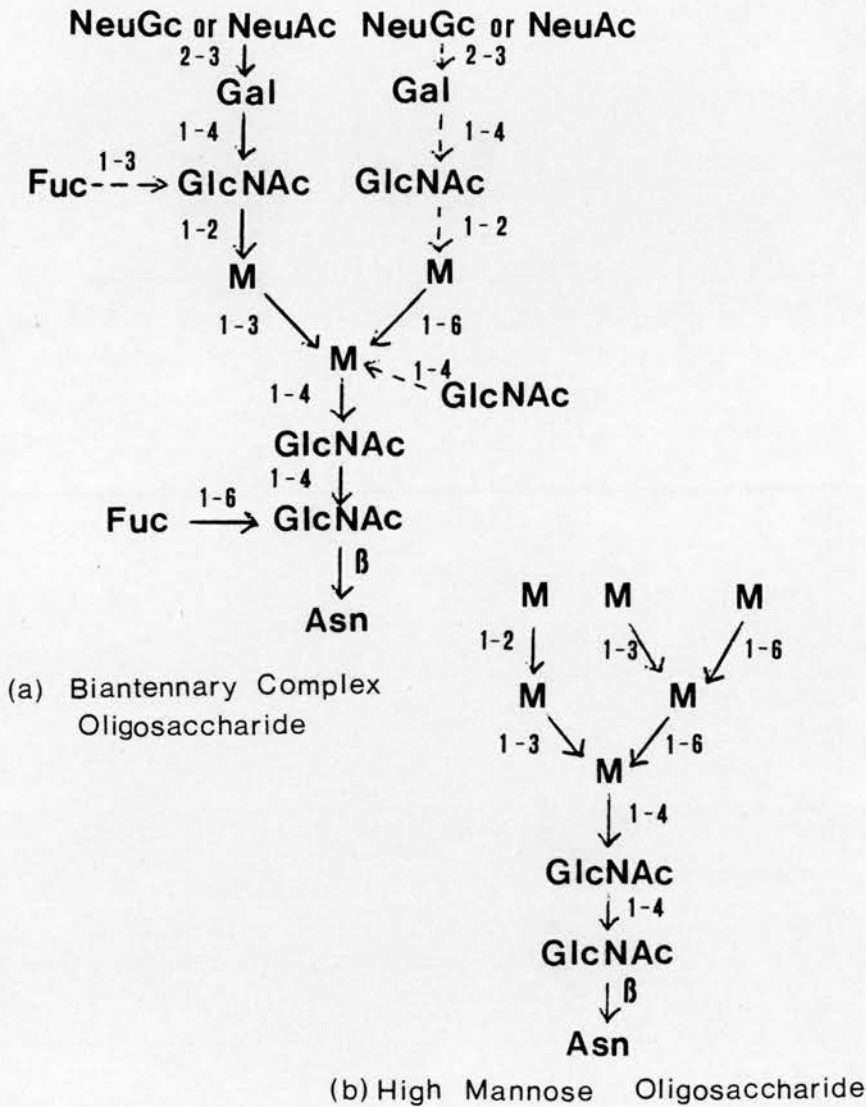


Figure 1.9 Structures of oligosaccharides in soluble form of dopamine β -hydroxylase. Dashed arrows indicate residues which are not present in all oligosaccharides. Data were obtained from Margolis et al. (1984). Asn, asparagine, Fuc, fucose, Gal, galactose; GlcNAc, N-acetylglucosamine; M, mannose; NeuAc, N-acetylneuraminic acid; NeuGc, N-glycolylneuraminic acid.

(Huber et al., 1979) and it has an affinity for a number of lectins (see table 1.4 and fig. 1.7), including Con A, WGA and LCL (Cahill and Morris, 1979; Gavine et al., 1984; Apps et al., 1985). The carbohydrate portion consists of residues of fucose, mannose, galactose, N-acetylgalactosamine, N-acetylglucosamine and sialic acid. Membrane and soluble forms of DBH have identical carbohydrate compositions (Fischer-Colbrie et al., 1982; Wallace et al., 1973; Geissler et al., 1977). The structures of the carbohydrate portion of soluble DBH are shown in figure 1.9. The oligosaccharide portion consists of biantennary oligosaccharides and high mannose oligosaccharides in a molar ratio of 2:1 and oligosaccharides are linked to asparagine by N-glycosidic bonds (Margolis et al., 1984). The lectin-binding properties of DBH were used to purify the enzyme. The soluble form of DBH was purified by chromatography of the lysate on Con A-Sepharose (Rush et al., 1974). This purification procedure was improved by chromatography of the lysate on DEAE - cellulose before chromatography on Con A-Sepharose (Winkler, 1982). Saxena and Fleming (1983) purified the membrane-bound form of DBH, by solubilization with octylglucoside followed by ion-exchange and exclusion chromatography. Fischer-Colbrie et al. (1982) solubilized membrane DBH with SDS and purified it by sequential affinity chromatography on Con A - and WGA-Sepharose columns.

Although there have been numerous studies on DBH, it is not known if DBH is composed of four

identical subunits or two types of subunit, and the relationship between the soluble and membrane-bound forms is also obscure. SDS gel electrophoresis of membrane DBH from bovine adrenal medulla showed that it contains two subunits of apparent Mr 70000 and 75000 (Saxena and Fleming, 1983). Membrane DBH from rat pheochromocytoma contains subunits of apparent Mr 77000 and 73000, whereas the soluble form is mostly made up of subunits Mr 73000 (Saban et al., 1983). Others (O'Connor et al.; 1979; Wallace et al., 1973) found one band on SDS polyacrylamide gels indicating that all the subunits are identical. As shown in figure 1.7, DBH is resolved into three spots on two dimensional gels (Gavine et al.; 1984). This may result from differences in the polypeptide chains and/or microheterogeneity of the carbohydrate portion. One of the branches of the biantennary oligosaccharide is variable (Fig. 1.9) indicating that the subunits are not identical (Margolis et al.; 1984), and the number of carbohydrate chains is not the same on each subunit.

Cyanogen bromide cleavage of membrane DBH produced more peptides than could be accounted for by its methionine content if it was assumed that only one type of polypeptide chain was present (Slater et al., 1981). Also digestion with trypsin produced more peptides than could be accounted for by its arginine plus lysine content (Sokoloff et al., 1985). These findings together with the observation that the N-terminal amino acid sequence is heterogeneous (Skotland et al., 1977), suggest that DBH is composed of non-identical polypeptide chains. DBH

contains 8 atoms Cu/tetramer (Ash et al., 1984) or 2 atoms Cu/subunit (Klinman et al., 1984). Klinman et al. (1984) propose that the two electrons required for the hydroxylation of dopamine (fig. 1.1) are donated by both copper atoms in each subunit. This would suggest that all the subunits are identical in function. Ultimate proof of non-identical subunits would require preparative separation and sequencing of the different subunits.

The relationship between the soluble and membrane-bound forms of DBH has been extensively investigated, without any firm conclusions. The soluble and membrane-found forms are similar in amino acid composition (Winkler, 1976), immunological properties (Aunis et al., 1975; Ljones et al., 1976), and carbohydrate composition (Fischer-Colbrie et al., 1982). However, temperature induced phase separation in Triton X-114 of chromaffin granule proteins is able to separate the two forms of DBH (Pryde and Phillips, 1986), and they behave differently in charge shift crossed immunoelectrophoresis and crossed hydrophobic interaction immunoelectrophoresis (Bjerrum et al., 1979; Skotland and Flatmark, 1979). Furthermore membrane DBH, but not soluble DBH, can be reconstituted into phospholipid vesicles (Saxena and Fleming, 1983). So the amphiphilic membrane DBH may have an extra hydrophobic portion to anchor it to the membrane. Arrhenius plots for the activities of soluble and membrane DBH are different (Aunis et al., 1977).

The biosynthetic relationship between the membrane-found and soluble forms of DBH is also controversial. DBH is synthesized on membrane-bound polysomes in bovine adrenal medulla and pheochromocytoma cells (Saban et al., 1983; Saban and Goldstein, 1984). In pheochromocytoma cells DBH is synthesized as a precursor of apparent Mr 67000 which is rapidly glycosylated to form a subunit Mr 77000. This is then converted to a subunit Mr 73000 within 15-90 minutes (Saban et al., 1983). Membrane form of DBH appears to be composed of both types of subunit whereas the soluble form consists predominantly of the subunit Mr 73000. Saban et al (1983) propose that soluble DBH in pheochromocytoma may arise from post-translational processing of membrane DBH. Others (Sokoloff et al., 1985; Ledbetter et al., 1978; Saxena and Fleming, 1983) have found no evidence for a precursor-product relationship between the two forms.

1.8.3 Glycoprotein II and glycoprotein III

These were identified by Huber et al (1979) together with DBH which was then called glycoprotein I. Glycoprotein II (GpII; Mr 86000-100000, pI 3.6-5.0) and glycoprotein III (GpIII; Mr 37000-43000, pI 4.6-5.3) are very heterogeneous, appearing as ill-defined bands on one-dimensional electrophoresis, and as a series of spots (see fig. 1.7) on two-dimensional gels (Gavine et al., 1984; Fischer-Colbrie et al., 1982; Pryde and Phillips, 1986). The wide range of pI may result, in part from incomplete sialylation (Fischer-Colbrie et al., 1982).

However, neuraminidase treatment of GpIII fails to remove all of its heterogeneity suggesting other forms of modification (Gavine et al., 1984). Both glycoproteins have an affinity for WGA and ConA (Fischer-Colbrie et al., 1982; Gavine et al., 1984). They were solubilized from chromaffin granule membranes with SDS and isolated by affinity chromatography on ConA - and WGA - Sepharose (Fischer-Colbrie et al., 1982). These glycoproteins contain 30% carbohydrate; mannose, galactose, N-acetyl-galactosamine and sialic acid are the main sugars. The high carbohydrate content probably explains why they stain poorly with Coomassie Blue. GpII is only found in the granule membrane but a soluble form of GpIII also occurs in the matrix (Fischer-Colbrie et al., 1982).

Antisera were raised against both the soluble and membrane-bound forms of GpIII and the distribution of GpIII in the adrenal medulla was determined immunologically by rocket immunoelectrophoresis (Fischer-Colbrie et al., 1984). GpIII is specifically confined to chromaffin granules and can be used as a marker for the granule membrane. The soluble form is secreted from perfused adrenal medulla during stimulation with carbamylcholine. An immunologically identical antigen was found in anterior and posterior pituitary (Fischer-Colbrie et al., 1984). The functions of GpIII and GpII are not known.

1.8.4 Glycoproteins H, J and K

Of the Con A - binding proteins identified by Gavine et al. (1984), glycoproteins H, J and K appear

to be among the major components not hitherto characterized. Glycoproteins H, J and K are major bands on an autoradiograph of a one-dimensional gel, after decoration with ^{125}I -labelled Con A, and are also major spots (see fig. 1.7) on a two-dimensional gel (Gavine et al., 1984; Pryde and Phillips, 1986). Presumably these glycoproteins contain less carbohydrate and the carbohydrate portions are less heterogeneous than those of GpIII. Glycoproteins H, J and K are integral membrane proteins but they may contain hydrophilic regions because of their behaviour on phase partition in Triton X-114 (Pryde and Phillips, 1986). The function of these glycoproteins is not known.

1.9 The project

Many proteins found in the chromaffin granule membrane have not been characterized because:

(1) contamination with cytosolic or matrix proteins makes it difficult to identify intrinsic membrane proteins; (2) intrinsic membrane proteins must be solubilized with a suitable detergent; (3) it is difficult to follow the purification of a protein which has no enzymic activity; (4) some enzymes are inactivated when they are solubilized from the membrane. The specificity of lectin binding gives a convenient route for the purification of membrane glycoproteins such as GpIII, J, K and DBH. Analysis of membrane proteins has been improved by the introduction of two-dimensional electrophoresis, and the transfer of separated proteins to cellulose nitrate sheets has enhanced the sensitivity

of antibody and lectin overlays. So, antibodies to purified membrane constituents can be used to investigate granule assembly and to locate membrane proteins in subcellular organelles and other tissues.

Many aspects of the structure and biosynthesis of DBH remain controversial. The membrane-bound form of the enzyme can be studied using monoclonal antibodies and specific polyclonal antibodies. Monoclonal antibodies also provide an powerful tool for the identification of membrane proteins that are hard to purify and have no known enzymic activity. Some leukemias, for example, have been diagnosed using monoclonal antibodies to antigens on the surface of lymphoid cells (Milstein, 1980). Two novel proteins were found in the chromaffin granule membrane using monoclonal antibodies SY38 and ASV48

CHAPTER TWO
MATERIALS AND METHODS

Materials

1. Chemicals and Biochemicals

Coomassie Brilliant Blue G-250, Coomassie Blue R, and urea were from Serva, Heidelberg, West Germany. Triton X-100 and Tween 80 were from Koch Light Laboratories Ltd. Octaethylene-glycol dodecyl ether was purchased from Kouyoh Trading Co. Tokyo, Japan; poly (acrylamide), carboxyl modified, was from Aldrich Chemical Co. Agarose for two-dimensional gels was from Litex, Denmark; ampholytes were from Bio-Rad Laboratories; dithiothreitol was from Boehringer. Bromophenol Blue, TEMED, sodium azide, Nonidet P40, sodium periodate and all other general chemicals were where possible of AnalaR grade and sodium dodecyl sulphate (specially pure), acrylamide (Electran) and N,N'-methylenebisacrylamide (Electran) were all obtained from BDH Chemicals.

Glycine, Hepes, Mes, Tris, Trizma base, Tween 20, Lubrol WX, Lubrol PX, 4-chloro-1-naphthol, PMSF, α -methyl-D-mannoside, N-acetylglucosamine, EDTA (disodium and tetrasodium salt) and 2-mercaptoethanol were all obtained from Sigma Chemical Co. Ltd.

2. Enzymes and Proteins

Wheat germ agglutinin, Lens culinaris lectin, concanavalin A, trypsin, trypsin inhibitor, horse heart cytochrome c (type III), and Staphylococcus aureus protein A were from Sigma. Bovine serum albumin was purchased from Boehringer.

3. Chromatography media

Sephadex G25 and Sepharose CL-4B were from Pharmacia,

U.K. Biogel P-6DG was from Bio-Rad Laboratories. Lens culinaris lectin-agarose and concanavalin A-agarose were from Sigma.

4. Other materials

Cellulose nitrate sheets were obtained from Schleicher and Schüll, Dassel, West Germany. GF/C filters were from Whatman. 1.5 ml microcentrifuge tubes with screw cap lids for centrifugation and freeze-drying of samples were purchased from Starstedt. Cassettes for electroelution were from ISCO.

Normal horse serum was from Gibco-Bio-Cult, Glasgow. Freund's complete adjuvant and incomplete adjuvant were from Difco Laboratories. Sheep anti-mouse IgG and normal sheep serum were from Scottish Antibody Production Unit. Polyethylene glycol for cell fusion was purchased from Boehringer.

Anti-mitochondrial F_1 -ATPase serum was the gift of Frl. Y. Rudin (Biocenter, Basel). Anti-cytochrome b-561 serum was the gift of Dr. L. Kilpatrick. $Na^{125}I$ was obtained from Amersham International.

Methods

2.1 Biochemical Assays

2.1.1 Coomassie Blue G dye-binding assay for protein

The assay described by Bradford (1976) responds to microgram quantities of protein. Detergents such as sodium dodecyl sulphate (SDS), Triton X-100 and octaethylene-glycol dodecyl ether ($C_{12}E_8$) interfere with the assay. A detailed study of the assay has been



published (Read and Northcote, 1981).

A stock solution of the dye was prepared by dissolving 25 mg of Serva Coomassie Blue G-250 in 25 ml of 96% v/v ethanol, then 50 ml of 85% v/v ortho-phosphoric acid was added. The solution was stirred for 60 min at room temperature and finally diluted to 100 ml with distilled water. The reagent was diluted 5-fold in distilled water for the assay. Bovine serum albumin (BSA) was used as the standard protein. A stock solution of 1% w/v BSA in distilled water was prepared; the concentration of BSA was determined by measuring the absorbance at 280 nm; using $A_{280}^{1\%} = 6.6$.

A standard curve was prepared using 1-10 μ g of BSA in 100 μ l of distilled water. Samples for protein estimation were prepared in the same way in microcentrifuge tubes. Diluted dye-reagent (1 ml) was added to each tube. After 30 min the absorbance at 595 nm was measured, in a glass cuvette, against a blank of 100 μ l of distilled water in 1 ml of the same dye-reagent. A graph of A_{595} against concentration of BSA is relatively linear between 2-8 μ g of BSA/100 μ l (standard curve).

2.1.2 Catecholamine assay

Catecholamines were assayed by the spectrophotometric method of Euler and Hamberg (1950). Standard solutions (final vol. 1 ml) containing 0-500 μ M adrenaline bitartrate were prepared and 1 ml of 1 M sodium acetate-acetic acid buffer, pH 6.0 was added to each solution. After the addition of 200 μ l of a 100 mM iodine solution in potassium iodide, the solutions were incubated for 3 min, then 400 μ l of 50 mM $\text{Na}_2\text{S}_2\text{O}_5$

was added to each. The absorbance at 530 nm was measured within 5 min of adding $\text{Na}_2\text{S}_2\text{O}_5$ and a standard curve was constructed.

Aliquots (42 μl) of fractions collected after centrifugation of crude chromaffin granules on a gradient of 0.5-2.0 M sucrose, were diluted to 1 ml with distilled water and assayed for adrenaline in the same way as the standards.

2.1.3 Cytochrome c oxidase

Cytochrome c oxidase (E.C.1.9.3.1) was assayed according to Mason et al (1973). Ferrocycytochrome c was prepared by adding a few crystals of sodium dithionite to 62 mg of horse heart cytochrome c dissolved in 1 ml of a buffer containing : 50 mM Mes, 100 mM KCl, 1mM EDTA, 0.0025% w/v BSA, pH 6.5, saturated with nitrogen. Excess dithionite was removed by passage through a Sephadex G25 column equilibrated with the same buffer (Yonetani and Ray, 1965). Ferrocycytochrome c was collected in 15 ml of buffer and diluted to 80 ml in assay buffer (50 mM Mes, 0.5% w/v Tween 80, 1 mM EDTA, pH 6.5, saturated with nitrogen). The assays were conducted at 30°C, in a thermostated Unicam SP 1800 spectrophotometer, in a medium containing 1 ml of 57 μM ferrocycytochrome c in assay buffer, 5 μl of fraction and 10 μl of 0.1 M KCN in 0.1 M Tris- SO_4 , pH 7.2. KCN was omitted from the reference cuvette. The activity of cytochrome c oxidase in the fractions was calculated using an extinction coefficient of $21 \times 10^6 \text{ cm}^2/\text{mol}$ at 550 nm (Massey, 1959).

2.2 Preparation of pure chromaffin granule membranes.

Fresh bovine adrenal glands (approximately 40 glands were obtained from the local slaughter house and were placed on ice within about 20 min of slaughter. All preparations were carried out at room temperature with the glands and buffers being kept on ice. All centrifuge procedures were carried out at 4°C. The adrenal medulla was dissected from the cortex and placed in 0.3 M sucrose buffered with 10 mM Hepes, pH 7.0. The tissue was minced and homogenized in 0.3 M sucrose, 10 mM Hepes-NaOH, pH 7.0 and centrifuged for 5 min at 4000 r.p.m. ($1540 g_{av}$) in a Beckman JA 14 rotor. The supernatant was centrifuged for 30 min at 14000 r.p.m. ($18900 g_{av}$) in the same rotor. The pellet, which contains crude granules and mitochondria, was resuspended by homogenization in 0.3 M sucrose, 10 mM Hepes-NaOH, pH 7.0 and centrifuged for 20 min at 15000 r.p.m. ($17600 g_{av}$) in a Beckman JA 20 rotor. Mitochondria form the upper layer of the pellet and were washed from the lower layer of crude granules with 0.3 M sucrose, 10 mM Hepes-NaOH, pH 7.0. The crude granule pellet was resuspended and centrifuged through 1.6 M sucrose, 10 mM Hepes-NaOH, pH 7.0 for 60 min at 45000 r.p.m. ($158000 g_{av}$) in a Beckman 45 Ti rotor. The pellet contains relatively pure granules (Smith and Winkler, 1967).

The granules were lysed in 400 ml of hypotonic medium (10 mM Hepes-NaOH, pH 7.0) and then centrifuged at 45000 r.p.m. in a Beckman 45 Ti rotor for 20 min. Pure membranes were obtained by centrifugation of the crude

membrane pellet through 1.0 M sucrose, 10 mM Hepes-NaOH, pH 7.0 for 30 min at 45000 r.p.m. in the same rotor (Apps and Schatz, 1979). The membranes were washed to remove sucrose by resuspending them in 10 mM Hepes-NaOH, pH 7.0 and pelleted by centrifugation at 45000 r.p.m. for 30 min. The membranes were resuspended in 6-9 ml of 10 mM Hepes-NaOH, pH 7.0, 1.0 mM dithiothreitol (DTT), 0.1 mM EDTA, at a final protein concentration of 7-10 mg/ml.

2.2.1 Granule lysate

Phenylmethanesulphonyl fluoride (PMSF; final concentration 0.2 mM) was added to 200 ml of the granule lysate obtained above. This was then dialysed overnight against 1000 vol. of 1 mM EDTA, 1 mM Hepes-NaOH pH 7.0, and was then freeze-dried and dissolved in 10 ml of distilled water (Pryde and Phillips, 1986).

2.2.2 Preparation of crude membranes from protease-digested chromaffin granules

Crude chromaffin granules (17 mg of protein/ml) in 0.3 M sucrose, 10 mM Hepes-NaOH, 1 mM DTT, 0.1 mM EDTA, pH 7.0 (final volume 10 ml), were incubated with trypsin (50 µg/ml) for 30 min, 1 h or 3 h at 20°C, with gentle agitation. Control granules were incubated for 3 h in the absence of trypsin, or for 3 h with trypsin (50 µg/ml) which had previously been inactivated by mixing with trypsin inhibitor (100 µg/ml). After incubation in the presence or absence of trypsin, the granules were treated with trypsin inhibitor (100 µg/ml), diluted to 60 ml with 0.3 M sucrose, 10 mM Hepes-NaOH, pH 7.0, 100 µM PMSF and then centrifuged at 15000 r.p.m. ($17600 g_{av}$) for 20 min

in a Beckman JA 20 rotor at 4°C. The pellets of crude granules were resuspended in 0.3 M sucrose, 10 mM Hepes-NaOH, 100 µM PMSF, pH 7.0 and granules were purified by centrifugation through 1.6 M sucrose, 10 mM Hepes-NaOH, 100 µM PMSF, pH 7.0 as described in section 2.2.

Membranes were prepared by lysis of the purified granules, and stored at -70°C in 10mM Hepes-NaOH, 1 mM DTT, 0.1 mM EDTA, 100 µM PMSF, trypsin inhibitor (100 µg/ml).

2.3 Electrophoresis

SDS polyacrylamide gel electrophoresis was performed using the buffer system of Laemmli (1970) and the modifications introduced by Douglas and Butow (1976) for slab gel work.

2.3.1 Apparatus

- (a) Slab gel stand and buffer reservoirs.
- (b) Two flat glass plates, one 19 x 16.5 x 0.3 cm, the other of the same dimensions but with a 13 x 2 x 0.3 cm slot cut out of the top.
- (c) Plastic spacers 21 x 1 x 0.1 cm.
- (d) Blank comb 12.6 x 2.6 x 0.1 cm with a 12.6 x 0.7 x 0.2 cm ridge on the top.
- (e) Combs 12.6 x 2.7 x 0.1 cm with a 12.6 x 1.2 x 0.2 cm ridge and teeth. Combs had 8 teeth (1.6 x 1.1 x 0.1 cm), 11 teeth (1.5 x 0.9 x 0.1 cm), or 16 teeth (1.6 x 0.6 x 0.1 cm).
- (f) Bulldog clips to hold the cassette together.

2.3.2 Assembly of the cassette

Spacers were inserted between the two plates, one on either side and one along the bottom. The plates were

clipped together, with two clips on either side and two clips along the bottom. Pressure from the clips was on the spacers and the bottom clips also act as a stand for the cassette, holding it vertical during the pouring of the gel. The spacers were sealed with 1.5% w/v molten agar.

2.3.3 Pouring and running the gel

Solution A: 1.5 M Tris-HCl, 8 mM disodium EDTA, 0.4% w/v SDS, pH 8.8.

Solution B: 30% w/v acrylamide, 0.8% w/v N,N' - methylene-bisacrylamide.

Solution C: 3% w/v polyacrylamide, 1 mM NaN_3 , 1mM NaF.

Solution D: 0.5 M Tris-HCl, 8 mM disodium EDTA, 0.4% w/v SDS, pH 6.5.

The separating gel (containing an exponential gradient of acrylamide of 6-10%, 6-15%, 7-15% or 8-15% w/v) was poured into the cassette up to a mark made about 1 cm below the depth of the comb to be used (one-dimensional gel) or 1 cm below the top of the plate with the slot cut out (two-dimensional gel).

Composition of the separating gel

	Percentage of acrylamide and volume of solution					
	6% (20 ml)	8% (20 ml)	10% (10 ml)	15% (10 ml)	7% (24 ml)	15% (12 ml)
Solution A	5.0	5.0	2.5	2.5	6.0	3.0
Solution B	4.0	5.3	3.3	5.0	5.6	6.0
Solution C	3.3	3.3	1.7	1.7	4.0	2.0
Water	7.7	6.4	2.5	0.8	8.4	1.0
TEMED	10 μl	10 μl	5 μl	5 μl	10 μl	5 μl

All volumes in the table are in ml unless stated otherwise.

The separating gel was chemically polymerized by the

addition of 0.07% w/v ammonium persulphate to the gel mixtures. The separating gel was pumped (Gilson minipuls two-way pump) into the cassette from a mixing chamber containing 10 ml of acrylamide solution (12 ml for a two-dimensional gel). This gel solution was diluted from a 20 ml reservoir (24 ml for a two-dimensional gel). The diluted mixture was delivered to the cassette at the same rate (1.5 ml/min) as it was diluted and the total pouring time was about 20 min. After the gel had been poured water saturated butan-2-ol was layered over the top of the separating gel to ensure that it polymerized with a straight top. Butan-2-ol was replaced with water once the gel had polymerized.

Composition of the stacking gel

	Volume (ml)
Solution D	2.0
Solution B	1.2
Solution C	1.3
Water	3.4
TEMED	10 μ l

The stacking gel contained 4.5% w/v acrylamide and was chemically polymerized by the addition of 0.16% w/v ammonium persulphate to the gel mixture. The cavity between the plates was dried with filter paper, filled with stacking gel solution and the comb was inserted. A stacking gel (1.5 cm deep) was poured for two-dimensional electrophoresis.

After the stacking gel had polymerized the comb clips and bottom spacer were removed. Excess agar was cleaned away and the glass plate, with the slot cut out, was greased. The cassette was clipped to the slab gel stand and the upper and lower reservoirs were filled with

electrode buffer (0.25 M Tris, 0.1 M glycine, 0.1% w/v SDS, 2 mM EDTA). Electrophoresis was carried out overnight (16 h) at the following constant voltages: 6-10% gels, 45-50 V; 6-15% and 8-15% gels, 50-55 V; 7-15% two-dimensional gels 70-80 V. A Shandon Vokam constant voltage power source was used.

2.3.4 Preparation of samples for electrophoresis

Membranes and material eluted from lectin-agarose columns were delipidated by precipitation with 10 vols. of acetone : ethanol (1:1, v/v) in Corex tubes, in an ice/salt bath for 3-12 h. After centrifugation at 15000 r.p.m. ($17600 g_{av}$) for 20 min in a Beckman JA 20 rotor, the supernatant was discarded and the pellet containing the precipitated protein dissolved in 0.125 M Tris-HCl, pH 6.5, 2 mM EDTA, 10% v/v glycerol, 5% w/v SDS, 0.001% w/v Bromophenol Blue and 0.5-1% v/v 2-mercaptoethanol.

Membranes from granules treated with protease were solubilized by heating for 4 min at 95°C in a mixture containing 50 mM potassium biphthalate, pH 4.0 (Bender *et al.*, 1971), 10% v/v glycerol, 0.001% w/v Bromophenol Blue. The mixture was cooled to room temperature and then 0.5% v/v 2-mercaptoethanol was added.

Other samples were dissolved in 0.125 M Tris-HCl, pH 6.5, 2 mM EDTA, 10% v/v glycerol, 5% w/v SDS, 0.001% w/v Bromophenol Blue and 0.5-1% v/v 2-mercaptoethanol.

Samples were loaded into slots in the stacking gel

using a Hamilton syringe.

2.3.5 Fixing and staining the gels

Gels were fixed in 10% v/v acetic acid, 20% v/v methanol for at least 30 min at 37°C. Then gels were stained with Serva Coomassie Blue R (0.25% w/v) in 7.5% w/v acetic acid, 50% v/v methanol for 5 min at 37°C, and destained in 7% v/v acetic acid, 10% v/v methanol at 37°C.

2.4 Two-dimensional electrophoresis

Two-dimensional electrophoresis was performed essentially as described by O'Farrell (1975).

2.4.1 First-dimension isoelectrofocussing gels

Glass tubes (14 x 0.3 cm) were sealed at the bottom with parafilm and filled to a depth of 11.5 cm with a gel mix containing: 4.5% w/v acrylamide, 0.06% w/v N,N'-methylenebisacrylamide, 9.5 M urea, 2% w/v Triton X-100, 2% v/v ampholytes (Bio-Rad 3-10), and 0.05% w/v ammonium persulphate to chemically polymerize the gel. Overlay buffer containing 5 M urea, 2% w/v Triton X-100, 1% v/v ampholytes (Bio-Rad 3-10) was layered over the polymerizing gel mixture.

Eluate from Lens culinaris lectin (LCL) - agarose was desalted using small columns of Biogel P-6DG. Chromaffin granule membranes, pure granules, eluate from LCL-agarose and sucrose gradient fractions were freeze-dried in 1.5 ml microcentrifuge tubes and dissolved in sample buffer, containing: 9.5 M urea, 4% w/v Triton X-100, 2% v/v ampholytes (Bio-Rad 3-10),

0.5% v/v 2-mercaptoethanol and 0.001% w/v Bromophenol Blue. Electroeluted glycoproteins were diluted with 2 vols. of sample buffer.

After the gels had polymerized, overlay buffer was shaken from the top of the gels, and the gels were mounted in the tank. The bottom of the gels (positive end) were immersed in 0.2% v/v orthophosphoric acid. Samples (100-300 μ g of protein) were loaded at the basic / negative end and 10 μ l of overlay buffer was layered over the samples. The tubes were filled to the top with 0.6% v/v ethanolamine.

Proteins were focussed for 20 min at 100 V, 30 min at 200 V, 19 h at 400 V and finally for 20 min at 800 V. Gels were removed from the tubes and soaked for 30 min in soaking buffer (3% w/v SDS, 0.05 M Tris-HCl, pH 6.5, 10% v/v glycerol, 0.005% w/v Bromophenol Blue). Gels can be frozen at -70°C in the absence of buffer before further use or run in the second-dimension straightaway.

2.4.2 Second-dimension SDS polyacrylamide gel

The Laemmli (1970) SDS buffer system was used and the separating gel contained a 7-15% exponential gradient of acrylamide. When the separating gel had polymerized the cassette was clipped to the slab gel stand and a stacking gel (1.5 cm deep) reaching the top inside plate, was poured. If the first-dimension gel had been stored at -70°C, it was thawed and soaked for 30 min in soaking buffer. A 1% w/v agarose solution in the same buffer was prepared. The first-dimension

gel was placed on top of the stacking gel and hot agarose solution was poured on top of the gel to seal it to the stacking gel. When the agarose had set the top and bottom reservoirs of the slab gel stand were filled with electrode buffer (0.25 M Tris, 0.1 M glycine, 0.1% w/v SDS, 2 mM EDTA and electrophoresis was performed at 70-80 V (constant voltage) for 16 h.

Gels were fixed in 10% v/v acetic acid, 20% v/v methanol for 3-4 h at 37°C to remove ampholytes. Gels were stained with Serva Coomassie Blue R (0.25% w/v) in 7.5% v/v acetic acid, 50% v/v methanol for 5 min at 37°C, and destained in 7% v/v acetic acid, 10% v/v methanol at 37°C.

2.5 Immune replicas

Solutions

(a) Transfer buffer: 0.025 M Na_2HPO_4 , 0.02% w/v SDS, 20% v/v methanol.

(b) BSA buffer: 3% w/v BSA, 0.05% w/v NaN_3 , 20 mM Tris-HCl, 0.9% w/v NaCl, pH 7.2.

(c) Horse serum buffer: 5% v/v heat inactivated horse serum, 2.5% w/v BSA, 0.05% w/v NaN_3 , 20 mM Tris-HCl, 0.9% w/v NaCl, pH 7.2.

(d) Tris/salt buffer: 20 mM Tris-HCl, 0.9% w/v NaCl, pH 7.2.

Electrophoretic transfer of proteins from SDS polyacrylamide gels to cellulose nitrate sheets (Towbin et al., 1979) was performed in an Electro-Blot tank, in transfer buffer, for 90 min at 0.8 amp. Cellulose nitrate replicas were washed with BSA

buffer for 60 min, then the replicas were incubated with antiserum diluted in horse serum buffer for 90 min. Anti-mitochondrial F_1 ATPase serum was diluted 50-fold, anti-GpIII serum and anti-glycoproteins J and K serum were diluted 50 - or 100 - fold; anti-membrane DBH and anti-cytochrome b-561 sera were diluted 200 - fold in horse serum buffer. The replicas were washed five times in Tris/salt buffer; the middle three washes contained 0.05% w/v Nonidet P40. Replicas were incubated with ^{125}I -labelled protein A (5 kBq/ml) in Tris/salt buffer for 60 min and then washed five times in Tris/salt buffer, three washes containing 0.05% w/v Nonidet P40. Replicas were dried and autoradiographed on Agfa-Gevaert RP-1 film at room temperature for 7-21 days, or at -70°C with Kodak X-Omatic intensifying screens for 1-14 days.

2.5.1 Decoration of cellulose nitrate replicas of gels with ^{125}I -labelled lectins (Con A, WGA and LCL)

Cellulose nitrate replicas of gels were washed with periodate-treated BSA (3% w/v, in Tris/salt buffer) for 4 h (Pryde and Phillips, 1986). For this purpose 4% w/v BSA in 0.1 M acetic acid was treated with 10 mM NaIO_4 for 6 h, at 20°C . Glycerol was added to 10 mM and then the pH of the solution was adjusted to 4.5 with 2 M Tris. The solution was dialysed against Tris/salt buffer containing 0.05% w/v NaN_3 .

The replicas were then washed five times in Tris/salt buffer and incubated overnight (16 h) with ^{125}I -labelled lectin (10 kBq/ml) in Tris/salt buffer.

Replicas were then washed nine times in Tris/salt buffer, the middle wash containing 0.1% w/v Nonidet P40 (this was omitted when WGA had been used).

Replicas were dried and autoradiographed on Agfa-Gevaert RP-1 film at room temperature for 7-21 days or at -70°C with Kodak X-Omatic intensifying screens for 2-7 days.

2.5.2 Decoration of cellulose nitrate replicas with Con A and horseradish peroxidase (HRP)

Decoration with Con A and HRP was essentially as described by Clegg (1982), although Triton X-100 was omitted from the buffers and 0.05% v/v Tween 20 included in one of the washing steps; 4-chloro-1-naphthol was used as the substrate for colour development (Apps *et al.*, 1985).

2.6 Radioiodination of lectins and Staphylococcus aureus protein A

S. aureus protein A and Con A were dissolved in 0.05 M Na_2PO_4 , 0.2 M NaCl, pH 7.2, and radioiodinated by the standard chloramine-T method (Morrison, 1980; McConahey and Dixon, 1980). The following were added to 50 μl of Na^{125}I (18.5 MB_q) in a 1.5 ml microcentrifuge tube : 100 μl of protein A or Con A (2 mg/ml) and 45 μl of chloramine-T (16 mg/ml). The solution was mixed and left for 2 min at 0°C. The reaction was stopped by the addition of 45 μl $\text{Na}_2\text{S}_2\text{O}_5$ (19 mg/ml) and 45 μl of 0.2 M NaI. Unreacted Na^{125}I was removed on small columns of Biogel P-6DG. BSA

(3 mg) was added to 200 μ g of ^{125}I -labelled protein A or Con A and radioactivity was counted in an LKB gamma counter. The final specific activity was $1-3 \times 10^7$ Bq/mg protein.

WGA and LCL were dissolved in 0.05 M Na_2PO_4 , 0.2 M NaCl, pH 7.2, containing 10 mM α -methyl-D-mannoside (LCL) or 10 mM N-acetylglucosamine (WGA), and were radioiodinated by the two-phase procedure of Tejedor and Ballesta (1982). They were purified by passage through a column of Biogel P-6DG (Gavine *et al.*, 1984). The final specific activity was $2-5 \times 10^6$ Bq/mg protein, depending on the lectin.

2.7 Coupling Con A, WGA and LCL to Sepharose CL-4B

Cyanogen bromide activation of Sepharose CL-4B was carried out at room temperature by the procedure of March *et al.* (1974) with some modifications. Sepharose CL-4B (10 g) was washed with water and then mixed with 30 ml of 1.3 M Na_2CO_3 , 6.7% v/v cyanogen bromide, 3.3% v/v acetonitrile. The mixture was stirred for exactly 2 min and then the Sepharose was washed with 500 ml of ice cold 0.1 M NaHCO_3 . Immediately after washing the Sepharose was added to 10 mg of lectin (Con A, WGA, LCL) dissolved in 10 ml of 0.05 M NaHCO_3 , 0.2 M NaCl. After gentle agitation for 24 h at 4°C, the uncoupled lectin was filtered off and the lectin-Sepharose conjugate was washed with water. Then the conjugate was mixed for 3 h at 4°C with 10 ml of BSA (4 mg/ml in 20 mM Tris-HCl, 0.9% w/v NaCl, pH 7.2). After filtration

and washing with 500 ml of 2 M NaCl, followed by 500 ml of 20 mM Tris-HCl, 0.9% w/v NaCl, pH 7.2, the Sepharose-bound lectin was stored in 20 mM Tris-HCl, 0.9% w/v NaCl, 0.1% w/v NaN_3 , pH 7.2 at 4°C. 70-80% of the lectin was coupled to Sepharose; this was estimated by measuring the A_{280} of lectin (1 mg/ml) in 0.05 M NaHCO_3 , 0.2 M NaCl and the A_{280} of the uncoupled lectin filtered off from the lectin-Sepharose conjugate.

2.8 Sucrose density gradients

Linear gradients of sucrose buffered with 10 mM Hepes-NaOH, pH 7.0 were poured into Beckman "Ultra-Clear" centrifuge tubes for the SW 40.1 Ti rotor. The total volume of the tubes is 13 ml and gradients of 12.3 ml volume were poured to leave room for a sample volume of 500 μl . Isopycnic density gradient centrifugation of crude and pure chromaffin granule membranes was performed on linear gradients of 0.4 - 1.6 M sucrose. Centrifugation of crude chromaffin granules was performed on a linear gradient of 0.5 - 2.0 M sucrose; a 'cushion' consisting of 1 ml of 2.5 M sucrose was placed at the bottom of the gradient to prevent granules from pelleting. A gradient mixer with two connected chambers of the same volume was used to pour the gradients. A solution containing the lower concentration of sucrose was pumped from the mixing chamber (A) onto the forming gradient, slowly enough to allow the solution in chamber B, containing the highest concentration of sucrose, to flow into chamber A. So, a sucrose solution of increasing

density was pumped into the centrifuge tube; the solution of lower density floating above that of higher density. Volumes and concentrations of sucrose in 10 mM Hepes - NaOH, pH 7.0 for the two mixing chambers were calculated according to the following formula.

$$C_B = C_A + \frac{V_t}{V_g} (C_{\text{bottom}} - C_{\text{top}})$$

where C_A = lower sucrose concentration.

C_B = higher sucrose concentration.

V_t = sum of volumes of the two mixing chambers.

V_g = total volume of the gradient.

C_{bottom} = concentration of sucrose at the bottom of the gradient.

C_{top} = concentration of sucrose at the top of the gradient.

2.8.1 Preparation of crude and pure membranes for density gradient centrifugation

Crude and pure chromaffin granule membranes were prepared as described in section 2.2. Membranes were diluted with 10 mM Hepes-NaOH, pH 7.4 and washed by centrifugation at 50000 r.p.m. ($171000g_{av}$) for 20 min in a Beckman 70.1 Ti rotor. The pellet was resuspended in 2 M sucrose, 10 mM Hepes-NaOH, pH 7.0 and the molarity of sucrose in the resulting suspension was calculated from the refractive index (measured using an Abbe '60 refractometer). Five hundred microlitres of pure membranes (2 mg of protein/ml) or crude membranes (4 mg of protein/ml) in 1.7 M sucrose, 10 mM Hepes-NaOH, pH 7.0 were pumped under a linear gradient of 0.4 - 1.6

M sucrose. Gradients were centrifuged to equilibrium at 35000 r.p.m. ($149800 g_{av}$) for 13 h in a Beckman SW 40.1 Ti rotor at 4°C.

2.8.2 Preparation of crude granules for density gradient centrifugation

Crude chromaffin granules were prepared as described in section 2.2 and 500 μ l of crude granules (30 mg of protein/ml) in 0.3 M sucrose, 10 mM Hepes-NaOH, pH 7.0, 1 mM DTT, 0.1 mM EDTA were layered over a linear gradient of 0.5 - 2.0 M sucrose. Gradients were centrifuged to equilibrium at 40000 r.p.m. ($195700 g_{av}$) for 3 h 30 min, or at 30000 r.p.m. ($110100 g_{av}$) for 14 h in a Beckman SW 40.1 Ti rotor at 4°C.

2.8.3 Fractionation of gradients

Gradients were fractionated at 4°C. The tube was pierced at the bottom with a needle and the gradient was pumped off from the bottom into 10, 12 or 21 fractions within 30 min. The concentration of sucrose in each fraction was determined, immediately after collection, from measurements with a refractometer (Abbe '60). Protein was determined by the method of Bradford (section 2.1.1). Fractions were stored at -15°C.

2.9 Solubilizing dopamine β -hydroxylase, glycoprotein III and glycoproteins J and K from freshly prepared chromaffin granule membranes.

Freshly prepared pure chromaffin granule membranes (2 mg of protein/ml) were suspended in 20 mM Hepes-NaOH, pH 7.2 containing 1% w/v octaethylene-glycol dodecyl

ether ($C_{12}E_8$). The suspension was vortex-mixed and left to stand at room temperature for 30 min. The suspension was stored overnight in 1.5 ml microcentrifuge tubes, surrounded by ice, in a refrigerator before being centrifuged at 20000 r.p.m. ($31300 g_{av}$) for 20 min in a Beckman 45 Ti rotor at $4^\circ C$. Solubilized membrane proteins were found in the supernatant and the pellet contained unsolubilized membrane material.

2.10 Partial purification of dopamine β -hydroxylase, glycoprotein III, and glycoproteins J and K by sequential affinity chromatography on *Lens culinaris* lectin-agarose and concanavalin A-agarose.

This method has been described (Wood et al., 1985). The whole procedure was carried out at $4^\circ C$. Columns ($5\text{cm} \times 0.5\text{ cm}^2$) packed with LCL-agarose and Con A-agarose were linked together in series with plastic tubing. After a wash with 100 ml of 20 mM Tris-HCl, 0.9% w/v NaCl, 0.1% w/v NaN_3 , pH 7.2 (= buffer 1) at a flow rate of 10 ml/h, they were equilibrated with 100 ml of 20 mM Hepes - NaOH, 0.5 M NaCl, 0.1% w/v $C_{12}E_8$, pH 7.2 (= buffer 2) at a similar flow rate.

Proteins were solubilized from chromaffin granule membranes as described in section 2.9. Solubilized proteins (7.5 ml) in 20 mM Hepes - NaOH, 1% w/v $C_{12}E_8$, pH 7.2 were diluted to 150 ml with 20 mM Hepes-NaOH pH 7.2 in order to reduce the $C_{12}E_8$ concentration to 0.05% w/v and passed (4.5 ml/h) through the LCL-agarose column. The exact amount of solubilized protein applied to the

column was not known since it was not possible to determine the protein concentration of the solubilized material by the Bradford method, due to the presence of $C_{12}E_8$. Material not adsorbed by LCL-agarose was immediately applied to a column of Con A-agarose. The columns were washed with 50 ml of buffer 2 at a flow rate of 4.5 ml/h and the effluent was pooled with the material passing through the Con A - agarose column.

Glycoproteins were eluted from the columns by removing LCL-agarose and Con A - agarose from the columns and mixing them with 8 ml of buffer 2 containing 10% w/v α -methyl-D-mannoside. The resulting suspensions were stirred for 2 h at 4°C and centrifuged at 3000 r.p.m. ($1636 g_{av}$) for 5 min in a MSE bench centrifuge. The supernatants containing eluted glycoproteins were collected. The pellets containing lectin-agarose were resuspended in 8 ml of buffer 2 containing 10% w/v α -methyl-D-mannoside. After stirring for 2 h at 4°C and centrifugation at 3000 r.p.m. for 5 min, the supernatants were collected and pooled with the previous supernatants. They were filtered through Whatman GF/C filters and then through cellulose nitrate filters (0.45 μ m pore size). The pellets containing LCL-agarose and Con A - agarose were resuspended in buffer 1 and re-packed into columns.

Material which passed through the columns and material which was eluted was dialysed for 3 days at 4°C against several changes of 1 mM disodium EDTA. After freeze-drying, the material was redissolved in a

minimum volume of distilled water. It was not possible to estimate the amount of protein present in the effluent and eluate samples due to the presence of $C_{12}E_8$. The effluent and eluate fractions were electrophoresed in SDS polyacrylamide gels to determine which proteins were adsorbed or passed through the columns.

2.11 Electroelution of dopamine β -hydroxylase, glycoprotein III, and glycoproteins J and K from SDS polyacrylamide gels.

SDS polyacrylamide gels containing 6-15% exponential gradients of acrylamide were poured. A single slot was made right across the top of the stacking gel and the eluate from LCL-agarose was loaded into the slot. The eluate from one column of LCL-agarose was loaded onto two gels to prevent overloading of the gels with protein. Electrophoresis was carried out overnight (16 h) at 50 V and the gels were fixed, stained, destained, and then soaked in water for 30 min to remove acetic acid. Gel slices containing DBH, GpIII, and glycoproteins J and K were cut out from the gels, using a razor. Glycoproteins were electroeluted from the gel slices in cassettes for 5 h at 80 V with 0.25 M Tris, 0.1 M glycine, 0.1% w/v SDS, 2 mM EDTA, as buffer. The eluted glycoproteins were dialysed for 24 h at 4°C against several changes of distilled water, freeze-dried and redissolved in a small volume of distilled water. It was not possible to estimate the protein concentration of electroeluted DBH, GpIII, glycoprotein J (GpJ) and glycoprotein K

(GpK) by the Bradford method since SDS was bound to the electroeluted glycoprotein. Electroeluted glycoproteins were re-electrophoresed in an SDS polyacrylamide gel containing a 6-15% exponential gradient of acrylamide to check purity.

2.12 Production of a monoclonal antibody against dopamine β -hydroxylase

2.12.1 Immunization

Pure chromaffin granule membranes (50 μ g) were injected subcutaneously into 4 sites on the back of a 12 week-old Balb/c mouse. Freund's complete adjuvant (50 μ l) was injected into a fifth site. Four weeks later an intravenous injection of 100 μ g of pure chromaffin granule membranes was given. Seven days after the intravenous injection the mouse was killed and the spleen was removed.

2.12.2 Fusion

The fusion and cloning were carried out by Dr.J. Haywood (Biochemistry Department). Lymphocytes from the spleen of a mouse immunized with chromaffin granule membranes were fused with NS1 myeloma cells according to Bastin et al (1982). Thymocytes were teased out of the thymus of a 6 week-old female Balb/c mouse. Cloning was also carried out by the method of Bastin et al (1982). Supernatants from cultures of hybridoma cells were stored at -15°C in 0.05% w/v NaN_3 .

2.13 Assays for monoclonal antibodies

2.13.1 Immunodotting assay

Solutions

(a) Tris/salt buffer: 20 mM Tris-HCl, 0.9% w/v NaCl, pH

7.2.

(b) Normal sheep serum/Tween 20 buffer: 1% w/v BSA, 1% v/v normal sheep serum, 0.1% w/v Tween 20, 0.05% w/v NaN_3 , 20 mM Tris-HCl, 0.9% w/v NaCl, pH 7.2.

(c) SAM-HRP buffer: 0.1% v/v sheep anti-mouse I_gG conjugated to horseradish peroxidase (SAM-HRP), 0.1% w/v BSA, 20 mM Tris-HCl, 0.9% w/v NaCl, pH 7.2.

(d) Chloronaphthol stain: 0.06% w/v 4-chloro-1-naphthol, 0.03% v/v hydrogen peroxide, 20 mM Tris-HCl, 0.9% w/v NaCl, pH 7.2.

All incubations were performed at room temperature. Small rectangles of cellulose nitrate (3 x 2 cm) were incubated with chromaffin granule membranes or lysate (2 mg of protein/ml in Tris/salt buffer containing 0.1% w/v NaN_3) for 18 h. After two washes in Tris/salt buffer, cellulose nitrate rectangles were incubated with normal sheep serum/Tween 20 buffer for 1 h. Then cellulose nitrate rectangles were washed twice in Tris/salt buffer containing 0.05% w/v Tween 20. Rectangles were placed on a moist sheet of blotting paper, 1 μl aliquots of supernatants from cultures of lymphocyte / NS1 hybridoma cells were dotted onto the cellulose nitrate, and the rectangles were left on moist blotting paper in a covered chamber for 3 h. After two washes in Tris/salt buffer, the middle wash containing 0.05% w/v Tween 20, rectangles were incubated with SAM-HRP buffer for 1 h. Rectangles were washed twice with Tris/salt buffer and then stained with chloronaphthol stain.

2.13.2 Decoration of cellulose nitrate replicas of gels with supernatants from cultures of lymphocyte/NS1 hybridoma cells

The solutions are described in the previous section (2.13.1), except for serum buffer which contained 5% v/v normal sheep serum or 5% v/v heat inactivated horse serum, 0.05% w/v NaN_3 , 20mM Tris-HCl, 0.9% w/v NaCl, pH 7.2. All incubations were performed at room temperature.

Proteins were transferred from SDS polyacrylamide gels to cellulose nitrate sheets or strips (Towbin *et al.*, 1979) in SDS-free transfer buffer (0.025 M Na_2HPO_4 , 20% v/v methanol) for 6 h at 0.8 amp. Cellulose nitrate replicas were washed with Tris/salt buffer containing 0.1% w/v Tween 20, for 1 h. After two washes in Tris/salt buffer containing 0.1% w/v Tween 20, replicas were incubated in serum buffer for 1 h. Replicas were incubated with supernatant from a culture of hybridoma cells (diluted 5-fold or 100-fold in serum buffer) for 15 h at room temperature. Controls were incubated for 15 h in serum buffer. After two washes in Tris/salt buffer containing 0.1% w/v Tween 20, replicas were incubated in SAM-HRP buffer for 4 h at room temperature. Replicas were washed twice with Tris/salt buffer containing 0.1% w/v Tween 20 and then stained with chloronaphthol stain.

2.14 Preparation of antisera against dopamine β -hydroxylase, glycoprotein III, and glycoproteins J and K

Electroeluted GpJ, GpK and GpIII (300 μl) were mixed with Freund's complete adjuvant (300 μl) and

injected subcutaneously into male guinea pigs on day 1. Booster injections starting on day 30 using Freund's incomplete adjuvant were repeated every month. Blood was obtained 10 days after the booster injections by cardiac puncture and allowed to clot at room temperature for 1 h, then at 4°C overnight (16 h). After centrifugation at 5000 r.p.m. (1960 g_{av}) in a Beckman JA 20 rotor for 10 min at 4°C, antisera were decanted, filtered through cellulose nitrate (0.45 μ m pore size), and stored at -70°C in 0.05% w/v NaN_3 . Antisera from the fourth bleeding were used for experiments.

Electroeluted membrane DBH (300 μ l) was mixed with an equal volume of Freund's complete adjuvant and injected subcutaneously into a New Zealand white female rabbit. One month later a booster injection of m-DBH in Freund's incomplete adjuvant was given and booster injections were repeated every month. Blood was obtained 10 days after the booster injections from the central ear artery and it was allowed to clot at room temperature for 1 h, then at 4°C overnight. After centrifugation and filtering, the antiserum was stored at -70°C in 0.05% w/v NaN_3 . Antiserum from the third bleeding was used for experiments.

Antiserum against GpIII was also raised in a New Zealand white female rabbit. Electroeluted GpIII (300 μ l) was mixed with Freund's complete adjuvant (300 μ l) and injected intradermally into a rabbit on days 1 and 14. A booster injection in Freund's incomplete

adjuvant was given on day 30 and the rabbit was bled from the central ear artery 10 days later. The blood was allowed to clot and was then centrifuged and filtered. Antiserum from the first bleed was used in experiments and it was stored at -70°C in 0.05% w/v NaN_3 .

2.15 Other Methods

2.15.1 Glassware cleaning

Chromic acid (100 ml) was prepared by the addition of 95 ml of concentrated H_2SO_4 to 5 ml of saturated aqueous potassium dichromate.

2.15.2 Dialysis

Visking dialysis tubing was boiled for 1 h in 1 mM tetrasodium EDTA and then in two changes of distilled water for 2 h. The tubing was washed with distilled water and stored at 4°C in 50% v/v ethanol. The tubing was washed with distilled water before use.

2.15.3 Scanning of gels and autoradiographs

Gels were stained, individual lanes were excised and scanned in a Gilford gel scanner (model 2520). The absorbance was measured at 550 nm. Autoradiographs were cut into slices, corresponding to single tracks of the gel, and slices were scanned in the same gel scanner. Absorbance was measured at 625 nm for autoradiographs.

2.15.4 Standard protein for electrophoresis

The molecular weight markers (Weber and Osborn, 1969) for electrophoresis were: β -galactosidase (Mr 128000); phosphorylase b (Mr 94000); bovine serum albumin (Mr 68000); ovalbumin (Mr 43000); carbonic anhydrase (Mr 30000);

trypsin inhibitor (Mr 21500); lysozyme (Mr 14300).

2.15.5 Radiolabelled standard proteins

^{14}C -labelled standard proteins were prepared by the method of Dottavio-Martin and Ravel (1978).

CHAPTER THREE

PURIFICATION OF DOPAMINE β -HYDROXYLASE,
GLYCOPROTEIN 111, GLYCOPROTEIN J AND
GLYCOPROTEIN K FROM BOVINE CHROMAFFIN GRANULE
MEMBRANES.

3.1 Identification of dopamine β -hydroxylase (DBH), glycoprotein 111 (Gp111), and glycoproteins H, J and K

These major glycoproteins of the chromaffin granule membrane can be identified on one-and two-dimensional gels either by Coomassie Blue staining or by decoration of cellulose nitrate replicas with ^{125}I labelled or HRP-conjugated lectins. The lectin-binding properties of the glycoproteins are summarized in table 3.1.

DBH was clearly visible in a one-dimensional gel stained with Coomassie Blue (fig. 3.1), migrating as a broad band with an apparent Mr of 75000 in the presence of 2-mercaptoethanol. Several other chromaffin granule proteins also migrate to this region. A two-dimensional electrophoretogram of chromaffin granule membranes is shown in figure 3.2 and DBH appears as a spot that is poorly focussed (Mr 70000-78000; pI 6.2-6.6). A replica of a two-dimensional gel of the eluate from LCL-agarose, decorated with ^{125}I -labelled Con A (fig. 3.10) shows that DBH migrated as three bands, called E, F and G by Gavine et al. (1984). It has been suggested (Skotland et al., 1977; Saxena and Fleming, 1983) that DBH is composed of two types of subunit, but the relationship between the subunits and the three bands is not clear. DBH was labelled by ^{125}I -labelled WGA and LCL as well as Con A after transfer to cellulose nitrate (fig. 3.1) and others (Huber et al., 1979; Fischer-Colbrie et al., 1982; Gavine et al., 1984; Pryde and Phillips, 1986) also report that DBH has an affinity for these lectins.

Table 3.1 Lectin-binding properties of chromaffin granule membrane glycoproteins

	LCL-agarose	Con A-agarose	Con A	WGA	LCL
Glycoprotein 11	++++	+	+++	++++	+
Dopamine β -hydroxylase	++++	+	++++	+	++
Glycoprotein H	++	++	++++	-	+
Glycoprotein J	+++	++	++++	-	-
Glycoprotein K	+++	++	++++	-	-
Glycoprotein IV	++	++++	++++	-	-
Glycoprotein III	+++	+	+	++++	+

Affinity for lectin-agarose and the strengths of labelling by different lectins are on the scale + (detectable to ++++ (very strong)

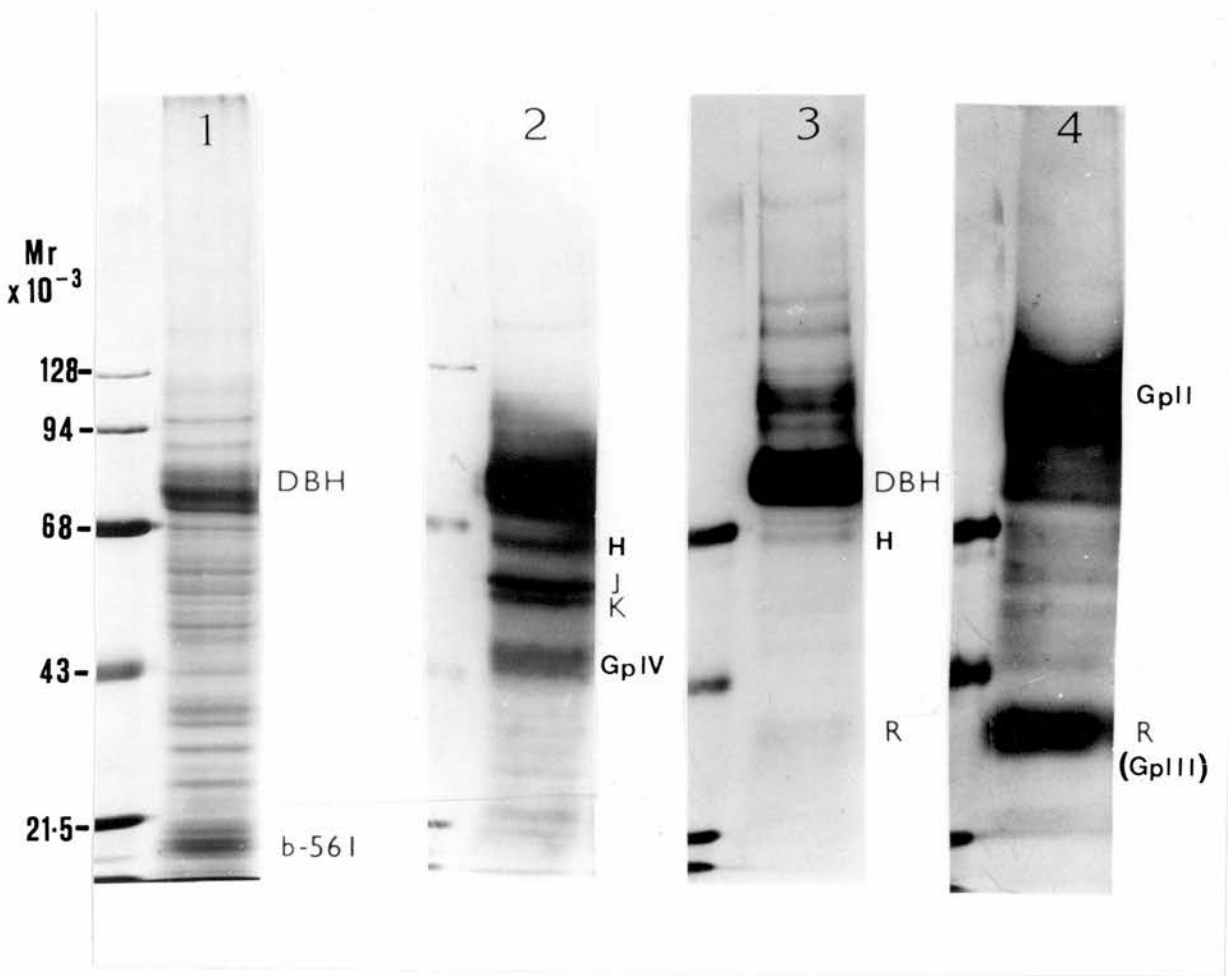


Figure 3.1 One-dimensional SDS polyacrylamide gels (6-10%) of chromaffin granule membranes. Track 1: stained with Coomassie Blue. Tracks 2-4: autoradiographs of cellulose nitrate replicas decorated with ¹²⁵I - labelled Con A (track 2), ¹²⁵I-labelled LCL (track 3) and ¹²⁵I - labelled WGA (track 4). Standard proteins were described in section 2.15.4. Abbreviations: DBH, dopamine β -hydroxylase; b-561, cytochrome b-561; GpII, glycoprotein II; GpIV, glycoprotein IV. Glycoprotein III (GpIII) of Huber et al. (1979) is also called glycoprotein R by Gavine et al. (1984).

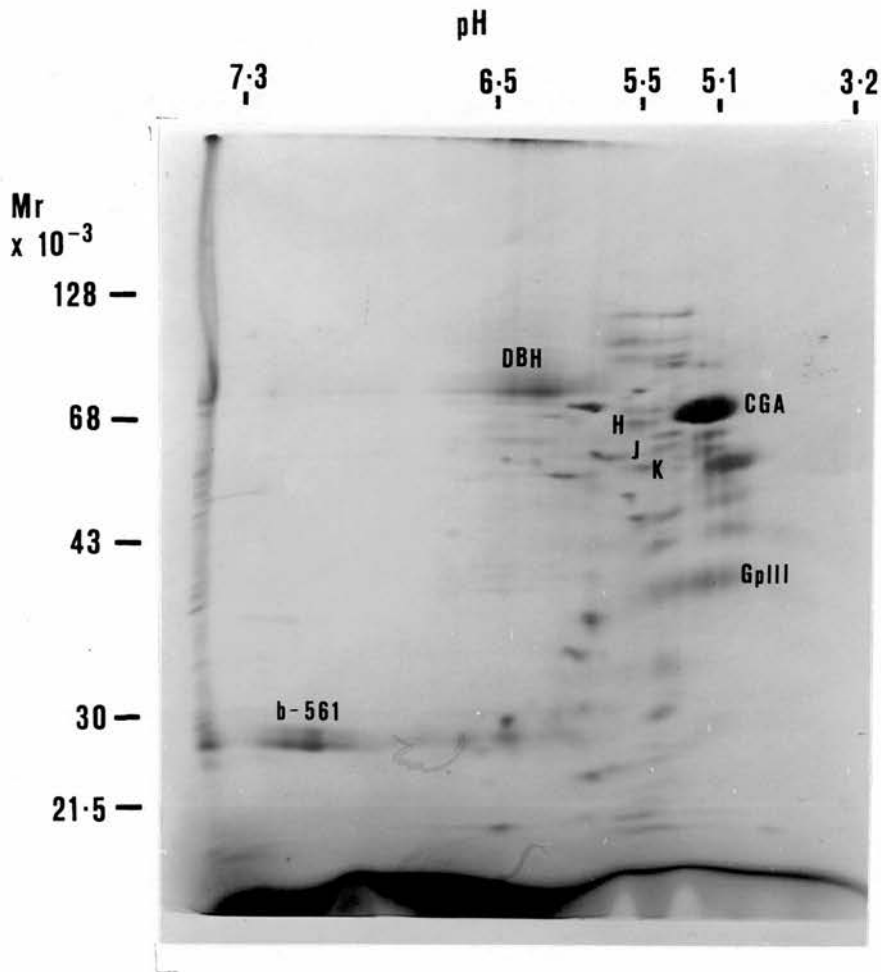


Figure 3.2 Two-dimensional electrophoretogram of chromaffin granule membranes (300 μ g), stained with Coomassie Blue. Abbreviations: DBH, dopamine β -hydroxylase; Gplll, glycoprotein lll; b-561, cytochrome b-561; CGA, chromogranin A

Glycoproteins H, J and K were difficult to distinguish in a one-dimensional gel of chromaffin granule membranes, stained with Coomassie Blue (fig. 3.1). However, these glycoproteins appeared as major bands in an autoradiograph of a cellulose nitrate replica decorated with Con A, and glycoprotein H (GpH) was also labelled by LCL (fig. 3.1). In order to identify glycoproteins H, J and K in a two-dimensional gel stained with Coomassie Blue, a cellulose nitrate replica of a stained gel was decorated with ^{125}I -labelled Con A and autoradiographed. Stained spots of proteins were visible on the replica and when the autoradiograph was superimposed on the replica; the intense compact spots labelled in figure 3.2 corresponded to glycoproteins H, J and K.

Gp111 (glycoprotein R of Gavine et al., 1984) migrated as a broad indistinct band in a one-dimensional gel (figs. 3.1, 3.7). Fischer-Colbrie et al. (1982) also report that Gp111 stains poorly with Coomassie Blue and they suggest that this reflects the high carbohydrate content of the glycoprotein. After transfer to cellulose nitrate, Gp111 was labelled by WGA and to a lesser extent by Con A and LCL (fig. 3.1); similar lectin-binding properties have also been reported by others (Fischer-Colbrie et al., 1982; Gavine et al., 1984; Pryde and Phillips, 1986). As shown in figure 3.2, Gp111 focussed over a broad pH range in two-dimensional SDS polyacrylamide gels (Mr 37000-40000; pI 4.8-5.3).

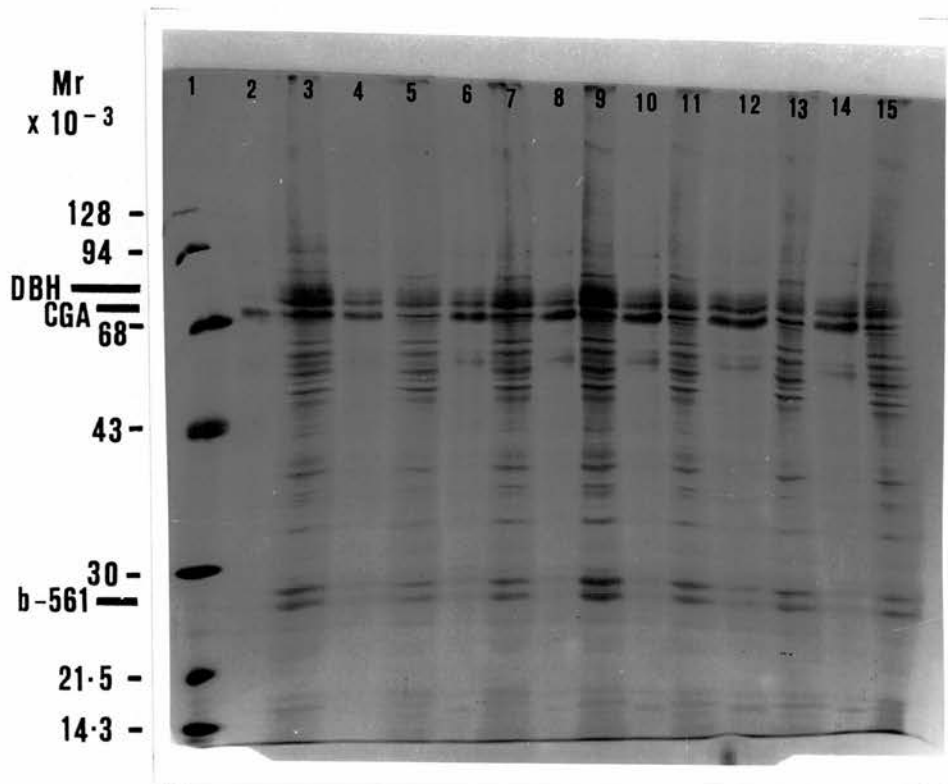


Figure 3.3 One-dimensional SDS polyacrylamide gel (8-15%) of proteins solubilized from frozen and thawed chromaffin granule membranes with Tween 20, Lubrol PX and Lubrol WX. Track 1, standard proteins were as in section 2.15.4. Track 2, proteins washed from membranes with 20 mM Hepes-NaOH pH 7.2. Track 3, untreated membranes. Proteins solubilized from frozen and thawed membranes by 2% w/v (track 4), 1% w/v (track 6) and 0.4% w/v Tween 20 (track 8); 0.2% w/v Lubrol PX (track 10); 1% w/v (track 12) and 0.4% w/v Lubrol WX (track 14). Membrane proteins not solubilized by 2% w/v (track 5), 1% w/v (track 7) and 0.4% w/v Tween 20 (track 9); 0.2% w/v Lubrol PX (track 11); 1% w/v (track 13) and 0.4% w/v Lubrol WX (track 15). Abbreviations: DBH, dopamine β -hydroxylase; b-561, cytochrome b-561; CGA, chromogranin A.

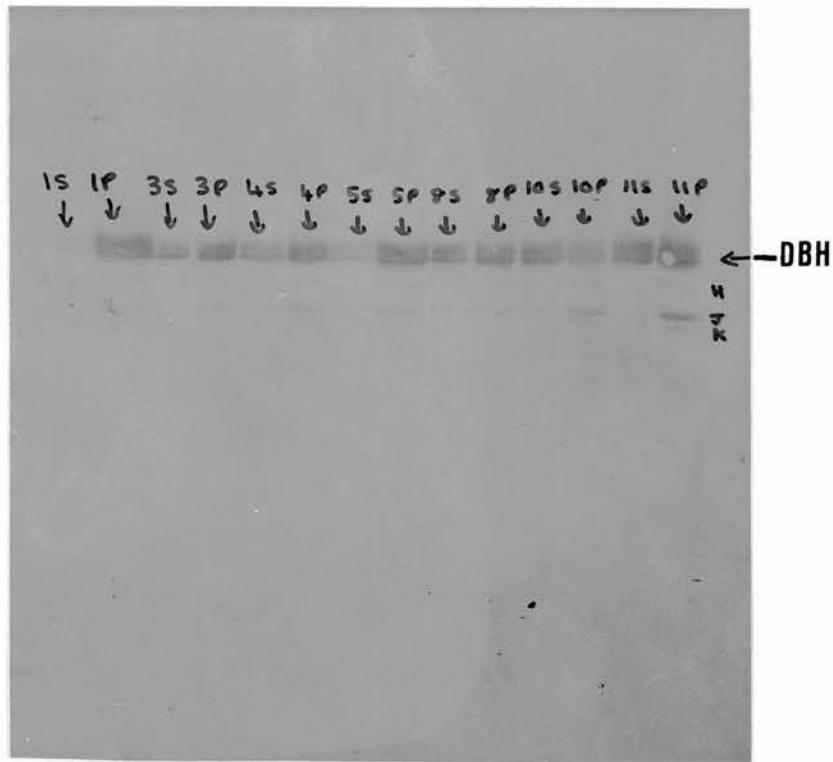


Figure 3.4 Cellulose nitrate replica decorated with ¹²⁵I-labelled Con A of proteins solubilized from frozen and thawed chromaffin granule membranes with Tween 20, Lubrol PX and Lubrol WX. Track 1S, proteins washed from chromaffin granule membranes with 20 mM Hepes-NaOH pH 7.2. Track 1P, untreated chromaffin granule membranes. Proteins solubilized from frozen and thawed chromaffin granule membranes with 2% w/v (track 3S), 1% w/v (track 4S), and 0.4% w/v Tween 20 (track 5S); 0.2% w/v Lubrol PX (track 8S); 1% w/v (track 10S) and 0.4% w/v Lubrol WX (track 11S). Membrane proteins that were not solubilized by 2% w/v (track 3P), 1% w/v (track 4P) and 0.4% w/v Tween 20 (track 5P); 0.2% w/v Lubrol PX (track 8P); 1% w/v (track 10P) and 0.4% w/v Lubrol WX (track 11P).

3.2 Solubilizing proteins from chromaffin granule membranes with detergent

Chromaffin granule membranes, which had been frozen at -15°C and then thawed, were suspended at 1 mg of protein/ml in 20 mM Hepes-NaOH, pH 7.2, and were solubilized with the following detergents: - Tween 80, Tween 20, Lubrol WX and Lubrol PX. A range of concentrations (0.1% - 2% w/v) was tried and unsolubilized material was pelleted by centrifugation at 20000 r.p.m. (31300 g_{av}) for 20 min in a Beckman 45 Ti rotor. Glycoproteins H, J, K and Gplll were difficult to identify in the gel stained with Coomassie Blue, illustrated in figure 3.3 but other gels showed that Gplll was not solubilized by Tween 80, Tween 20, Lubrol WX and Lubrol PX. A replica decorated with ^{125}I -labelled Con A of solubilized and unsolubilized proteins (fig. 3.4) shows that glycoproteins H, J and K were also not solubilized from chromaffin granule membranes by these detergents. Some DBH appeared in the supernatants (figs. 3.3, 3.4); this may have been soluble DBH washed from the surface of the membranes rather than detergent - solubilized membrane DBH.

Freshly-prepared or frozen and thawed chromaffin granule membranes (2 mg of protein/ml) were treated with 1% w/v or 2% w/v octaethylene-glycol dodecyl ether (C_{12}E_8); glycoproteins H, J, K and Gplll were solubilized in addition to DBH (figs. 3.5, 3.6, 3.7). Although not all of the proteins are clearly seen in figure 3.5 (track 4) and figure 3.7 (track 2), DBH, glycoproteins H, J and K are clearly visible

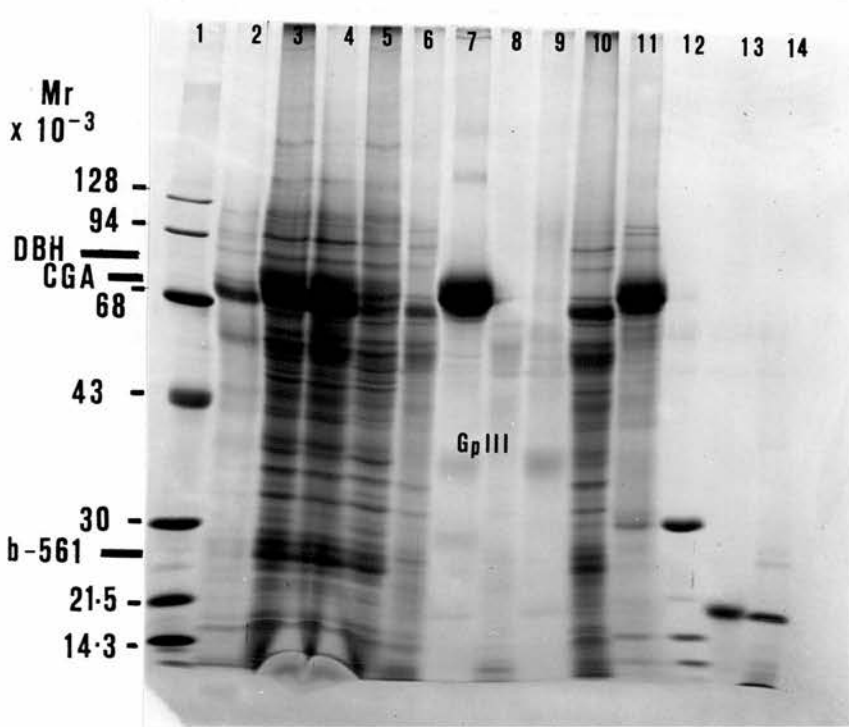


Figure 3.5 One-dimensional gel (6-15%), stained with Coomassie Blue, of fractions obtained from chromatography of solubilized chromaffin granule membrane proteins on lectin-Sepharose columns. Track 1, standard proteins were as in section 2.15.4. Track 2, proteins washed from the membranes with 20 mM Hepes-NaOH pH 7.2. Track 3, untreated chromaffin granule membranes. Track 4, proteins solubilized from freshly prepared chromaffin granule membranes with 2% w/v $C_{12}E_8$. Track 5, residual membrane proteins. Proteins not adsorbed by LCL-Sepharose (track 6), WGA-Sepharose (track 8), and Con A-Sepharose (track 10). Glycoproteins eluted from LCL-Sepharose (track 7), WGA-Sepharose (track 9), and Con A-Sepharose (track 11). Con A (track 12), WGA (track 13) and LCL (track 14). Abbreviations are as in Fig. 3.1.

Columns of lectin-Sepharose were linked together in series with plastic tubing (see p.90).

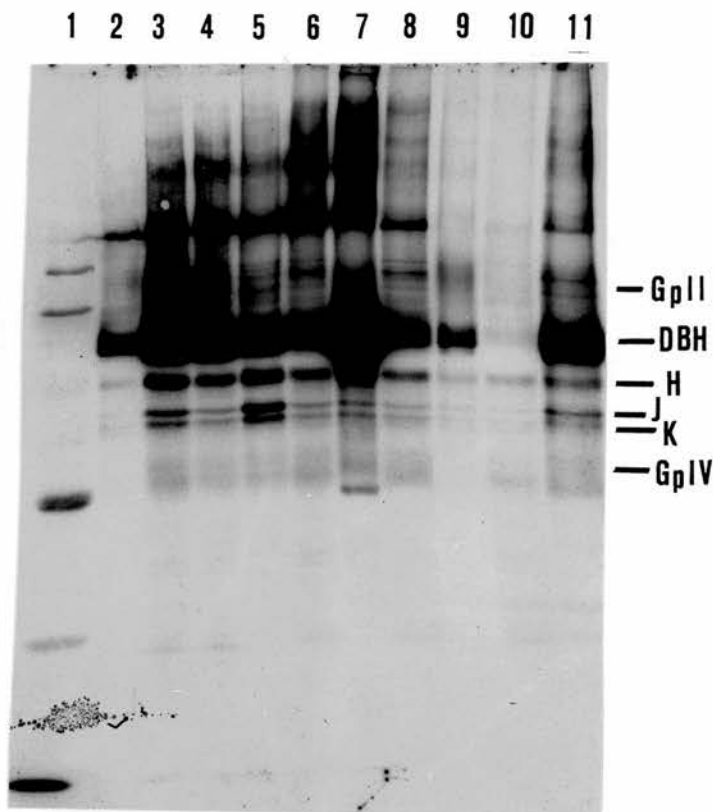


Figure 3.6 Replica decorated with ^{125}I -labelled Con A of fractions obtained from chromatography of solubilized chromaffin granule membrane proteins on lectin-Sepharose columns. Track 1, ^{14}C -labelled standard proteins were as in section 2.15. Track 2, proteins washed from membranes with 20 mM Hepes-NaOH pH 7.2. Track 3, untreated chromaffin granule membranes. Track 4, proteins solubilized from freshly prepared chromaffin granule membranes with 2% w/v C_{12}E_8 . Track 5, residual membrane proteins. Proteins not adsorbed by LCL-Sepharose (track 6), WGA-Sepharose (track 8), and Con A-Sepharose (track 10). Glycoproteins eluted from LCL-Sepharose (track 7), WGA-Sepharose (track 9), and Con A-Sepharose (track 11). Abbreviations are as in fig. 3.1

Columns of lectin-Sepharose were linked together in series with plastic tubing (see p.90).

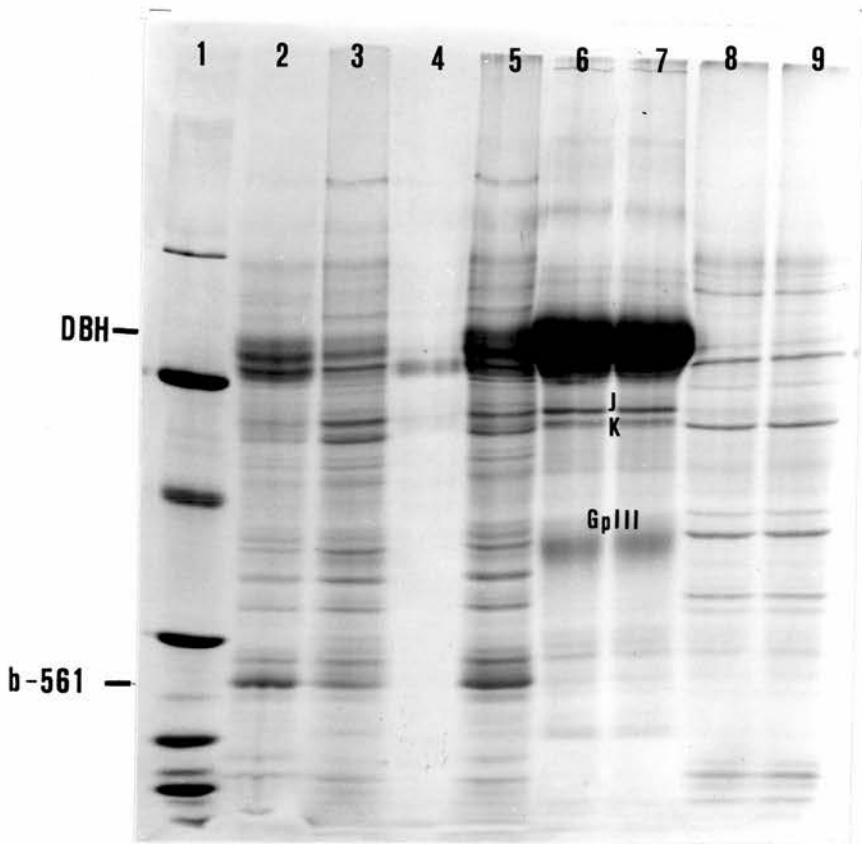


Figure 3.7 One-dimensional SDS polyacrylamide gel (6-15%) stained with Coomassie Blue. Track 1, standard proteins were as in section 2.15.4. Track 2, proteins solubilized from freshly prepared membranes by 1% w/v $C_{12}E_8$. Track 3, residual membrane proteins. Track 4, proteins washed from membranes by 20 mM Hepes-NaOH pH 7.2. Track 5, membranes after washing with 20 mM Hepes-NaOH pH 7.2. Tracks 6 and 7, glycoproteins eluted from LCL-agarose. Tracks 8 and 9, glycoproteins eluted from Con A-agarose. Abbreviations are as in Fig. 3.1

Columns of LCL-agarose and Con A-agarose were linked together in series (see section 2.10).

in figure 3.6 (track 4) and subsequent experiments with lectin columns establish their presence (see below). Fig. 3.7 also shows (track 4) that washing the membranes with buffer in the absence of detergent removes no protein other than residual chromogranin A. An attempt was made to increase the amount of protein solubilized by pretreating the membranes with 2% w/v Tween 20; this was not successful, possibly because micelle formation by Tween 20 prevented efficient solubilization of membrane proteins by $C_{12}E_8$.

In general, more protein was solubilized from fresh membranes than from frozen and thawed membranes, so whenever possible the former were used for glycoprotein purification. The procedure for solubilizing DBH, GpJ, GpK and Gplll from freshly-prepared membranes is described in section 2.9.

3.3 Partial purification of dopamine β -hydroxylase, glycoprotein lll, glycoprotein J and glycoprotein K by sequential affinity chromatography on LCL- and Con A - agarose columns.

In preliminary experiments WGA, LCL and Con A were coupled to Sepharose CL-4B by the procedure described in section 2.7 and columns (5 cm x 0.5 cm²) of these materials were equilibrated with 20 mM Hepes-NaOH, 0.5 M NaCl, 0.1% w/v $C_{12}E_8$, pH 7.2 (=buffer 2 in section 2.10). Proteins were solubilized from frozen and thawed chromaffin granule membranes (2 mg of protein/ml) by 2% w/v $C_{12}E_8$, diluted 20-fold in 20 mM Hepes-NaOH pH 7.2, and applied to separate columns of LCL-, WGA- and Con A-Sepharose at a

flow rate of 20 ml/h. After washing the columns with buffer 2, the columns were eluted with buffer 2 containing 10% w/v α -methyl -D- mannoside (Con A- and LCL - Sepharose) or 10 mg/ml of N-acetylglucosamine (WGA-Sepharose), and fractions were collected. The absorbance of these fractions at 280 nm was measured to determine relative protein concentrations. The results indicated that the glycoproteins were eluted gradually from the columns and not in sharp peaks. Glycoproteins were therefore subsequently eluted from lectin-Sepharose and lectin-agarose 'batchwise', after removing the materials from the columns.

Some DBH was bound by Con A- and LCL - Sepharose, but glycoproteins H, J, K and Gplll were not extensively bound by any column ^{at fast flow rates}. Possibly this was because the flow rate was too fast; optimal binding of glycoproteins to LCL-agarose and Con A - agarose occurred only when the flow rate was reduced to 4.5 ml/h.

In further experiments, the columns of lectin-Sepharose were linked together in series with plastic tubing. Proteins were solubilized from freshly-prepared chromaffin granule membranes (2 mg of protein/ml) by 1% w/v $C_{12}E_8$ as described in section 2.9, diluted 20-fold in 20 mM Hepes-NaOH, pH 7.2, and applied to a column of LCL-Sepharose at a flow rate of 4.5 ml/h. Material not adsorbed by LCL-Sepharose was immediately applied to WGA - Sepharose, and material not adsorbed by WGA-Sepharose was applied to Con A - Sepharose. The columns were then washed with buffer 2 at a flow rate of 4.5 ml/h. Glycoproteins were eluted from lectin-Sepharose 'batchwise', by removing the material from the columns and mixing it with buffer 2

containing the hapten sugar.

As shown in figure 3.5 Gp111 was bound both by LCL-Sepharose and by WGA-Sepharose. Most of the solubilized DBH was bound by LCL-Sepharose, very little by WGA-Sepharose and any DBH not retained by either column was bound by Con A - Sepharose. Glycoproteins H, J and K are difficult to identify in the gel stained with Coomassie Blue shown in figure 3.5. A Con A overlay of the glycoproteins eluted from the columns (fig. 3.6) shows that glycoproteins H, J, K and a large amount of DBH were adsorbed by LCL-Sepharose. GpH is difficult to see in figures 3.5 and 3.6 (track 7) because it migrates to a region of the gel only slightly below DBH and this in fact prevented subsequent purification of this glycoprotein (see later). The GpH that was not adsorbed by LCL-Sepharose was bound by WGA- and Con A - Sepharose (fig. 3.6) and figure 3.1 also shows that GpH has an affinity for these lectins.

The Con A overlay in figure 3.6 illustrates that residual GpJ and GpK were bound by Con A-Sepharose but much less DBH ^{remained to be} bound by this column than by LCL-Sepharose. GpJ and GpK were not labelled by LCL after transfer to cellulose nitrate (fig. 3.1) and the glycoproteins were not clearly visible in a Coomassie Blue stained gel of the eluate from LCL-Sepharose (fig. 3.5, track 7), so LCL probably has a weak affinity for GpJ and ^{after transfer to cellulose nitrate} GpK. The LCL-Sepharose probably became saturated with DBH (figs. 3.5, 3.6) which would thus reduce the binding of other glycoproteins to the column. A small amount of WGA, LCL and Con A leaked from the columns during these

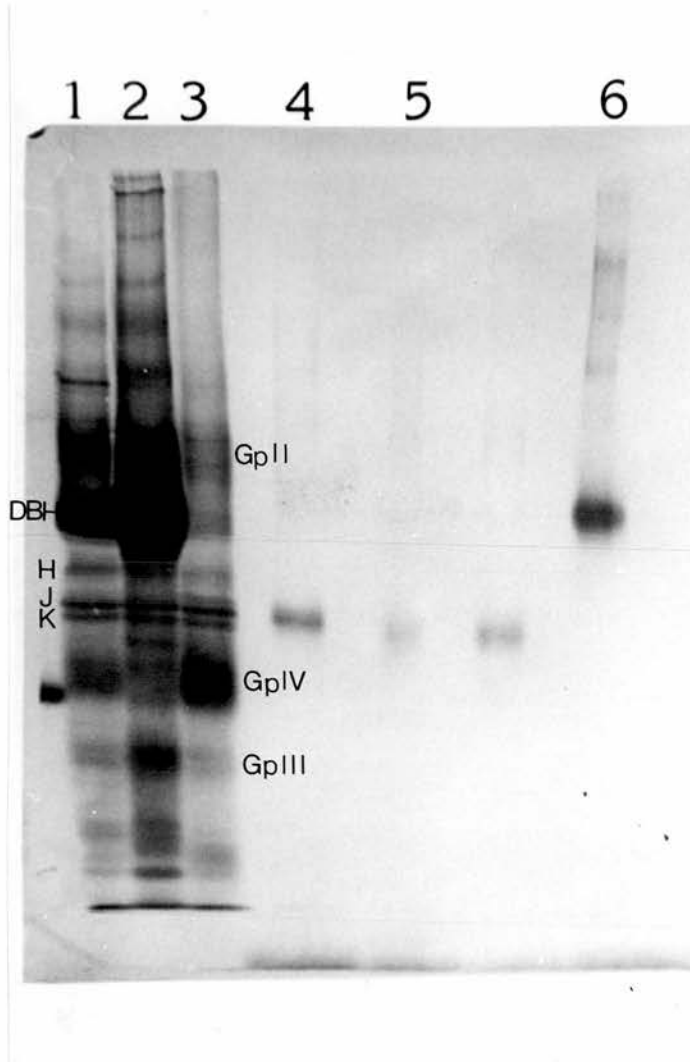


Figure 3.8 Cellulose nitrate replica decorated with Con A and horseradish peroxidase. Proteins were separated by one-dimensional electrophoresis (acrylamide concentration 6-10%). Track 1, chromaffin granule membranes; track 2, eluate from LCL-agarose; track 3, eluate from Con A-agarose; track 4, electroeluted glycoprotein J; track 5, electroeluted glycoprotein K; track 6, electroeluted dopamine β -hydroxylase.

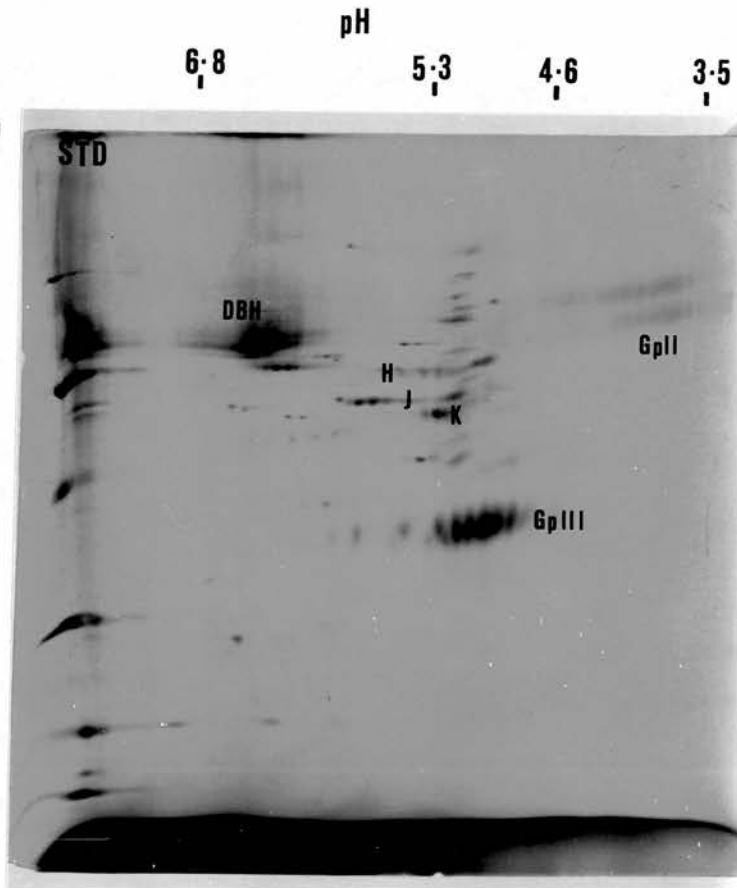


Figure 3.9 Two-dimensional electrophoretogram of glycoproteins eluted from LCL-agarose, stained with Coomassie Blue. STD, standard proteins (one-dimension) were as in section 2.15.4.

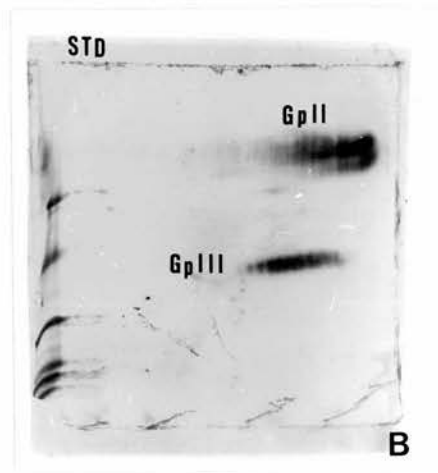
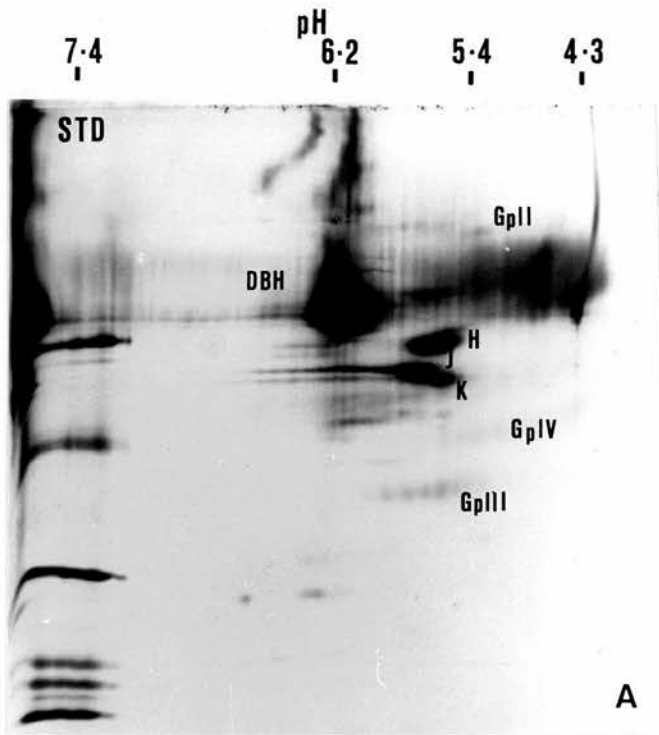


Figure 3.10 Two-dimensional gels of glycoproteins eluted from LCL-agarose. Autoradiographs of cellulose nitrate replicas decorated with (A) Con A and (B) WGA. STD, ^{14}C -labelled standard proteins (one-dimension).

experiments and these free lectins were detected in electrophoretograms of material which passed through the columns and in the eluate fractions (see fig. 3.5). Incomplete coupling of LCL to Sepharose might also partly explain why GpJ and GpK were bound weakly by this column; LCL-agarose and Con A-agarose, purchased from Sigma and used in subsequent work, were found to be more satisfactory.

The procedure for the partial purification of DBH, GpJ, GpK and GpIII is described in section 2.10 and by Wood et al. (1985). Glycoproteins eluted from LCL - and Con A-agarose were analysed by one-dimensional electrophoresis (fig. 3.7, tracks 6-9, Coomassie Blue; fig. 3.8, tracks 2-3, decoration with Con A and horseradish peroxidase after transfer to cellulose nitrate). Glycoproteins eluted from LCL-agarose were further characterized by two-dimensional electrophoresis and decoration of replicas with Con A and WGA (figs. 3.9, 3.10). Comparison with the work of Gavine et al. (1984) and Pryde & Phillips (1986) confirms that the Con A - binding glycoproteins II, IV, H, J and K and the WGA - binding glycoproteins II and III were eluted from the LCL-agarose column. GpH was not visible on a Coomassie Blue stained gel of the eluate from LCL-agarose (fig. 3.7, track 6) so GpH could not be purified by electroelution after affinity chromatography on LCL-agarose.

The glycoproteins bound by LCL- and Con A-agarose and their lectin-binding properties on cellulose nitrate replicas are summarized in table 3.1. LCL is specific

for α -D-mannose, the carbohydrate portion of DBH (see fig. 1.9) contains high mannose oligosaccharides (Margolis et al., 1984), which presumably explains why DBH binds to LCL-agarose. Glycoproteins H, J and K have a high affinity for Con A (Gavine et al., 1984) and were adsorbed by LCL-agarose suggesting that they also contain high mannose oligosaccharides.

Although Con A and LCL are reported to have the same sugar specificity (table 1.3), Gp111 was bound by LCL-agarose eventhough it has a low affinity for Con A. (Fischer-Colbrie et al., 1982; Gavine et al., 1984; Pryde and Phillips, 1986) as demonstrated in figs. 3.1 and 3.10. In general, Con A always decorated more glycoproteins after transfer of chromaffin granule membrane proteins to cellulose nitrate sheets (fig. 3.1, table 3.1) indicating that the sugar specificity of LCL differs slightly from that of Con A.

Glycoproteins 11, IV, J and K were adsorbed by the Con A-agarose column (fig. 3.8, table 3.1) although the DBH solubilized from the membranes was almost quantitatively bound to LCL-agarose. LCL-agarose bound much more GpJ and GpK than did LCL-Sepharose (compare fig. 3.5, track 7 with fig. 3.7, track 6). However, LCL-agarose did not retain DBH, GpJ and GpK quite quantitatively, suggesting that LCL-agarose has a weaker affinity for glycoproteins J and K than for DBH. Also, many of the binding sites on LCL-agarose were probably saturated with DBH which would result in less GpJ and GpK binding to the column.

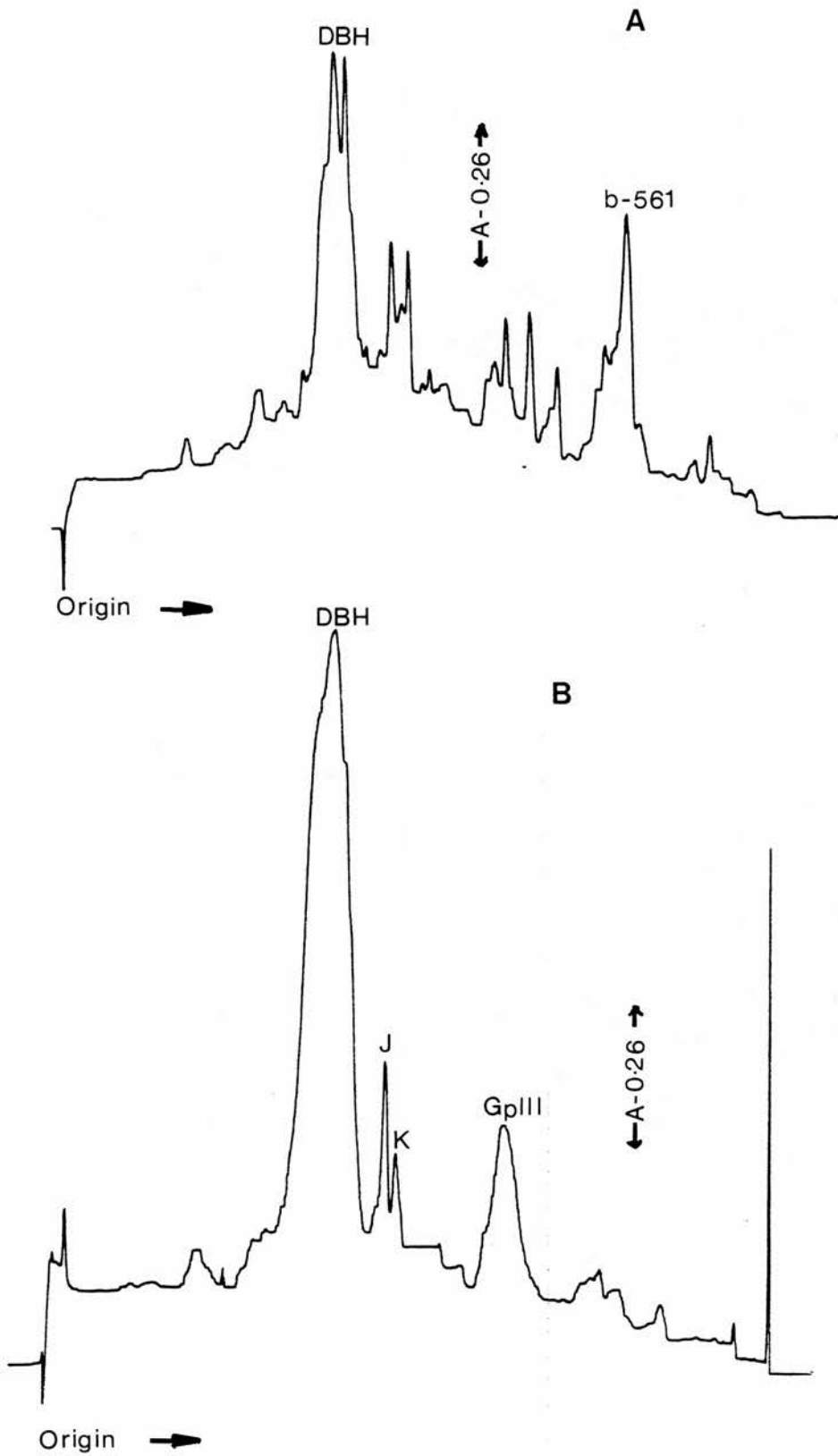


Figure 3.11 6-15% polyacrylamide gel of (A) chromaffin granule membranes and (B) eluate from LCL-agarose. Electrophoresis was performed under reducing conditions. Abbreviations: DBH, dopamine β-hydroxylase; GpIII, glycoprotein III; b-561, cytochrome b-561.

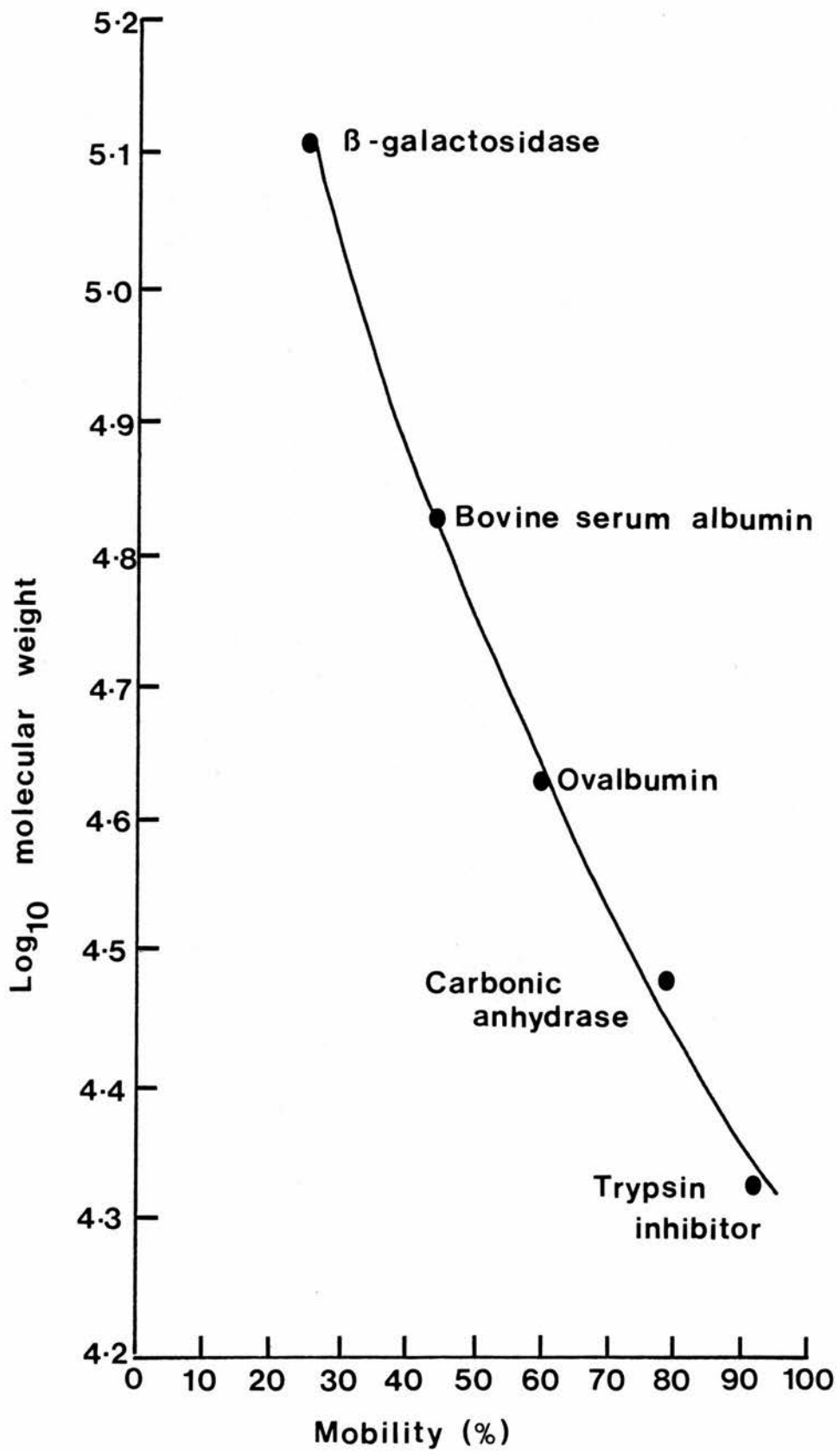
A number of proteins were eluted from Con A-agarose that were not labelled by Con A after transfer to cellulose nitrate sheets (see figs. 3.7, 3.8). Some of these proteins migrate with the same apparent Mr as the subunits of ATPase I (Percy et al., 1985) and the eluate from Con A-agarose contained ATPase activity (J. Percy personal communication). These proteins may bind to Con A-agarose rather non-specifically by hydrophobic interactions in spite of (or perhaps because of) the presence of 0.5 M NaCl in the buffer used to equilibrate and wash the columns.

3.4 Estimation of the apparent molecular weight of dopamine β -hydroxylase, glycoprotein lll and glycoproteins J and K

For estimations of apparent molecular weight, glycoproteins eluted from LCL-agarose and marker proteins of known molecular weight were separated in a 6-15% exponential polyacrylamide gel in the presence of 2-mercaptoethanol. Relative mobilities of the proteins were calculated from densitometric scans of individual gel tracks. Scans from a 6-15% gel of chromaffin granule membranes and the eluate from LCL-agarose are shown in figure 3.11. Apparent molecular weights of DBH, GpJ and GpK were calculated using the calibration curve shown in figure 3.12. DBH and Gplll migrated as very broad bands and the apparent molecular weights of these proteins were: DBH (range, 69000-80000; centre of peak 74000) and Gplll (range, 35000-40000; centre of peak 37000). The

Figure 3.12 Estimation of the apparent molecular weight of dopamine β -hydroxylase, glycoproteins J and K and glycoprotein lll. Polypeptides of known molecular weight were used to calibrate a 6-15% gel under reducing conditions. Five marker proteins were run together in one track of the gel. The molecular weight markers* were: β -galactosidase (Mr 128000); bovine serum albumin (Mr 68000); ovalbumin (Mr 43000); carbonic anhydrase (Mr 30000); trypsin inhibitor (Mr 21500). (*Weber and Osborn, 1969).

Figure 3.12



apparent molecular weight of GpJ was 57000 and that of GpK 53000. These results are similar to the apparent molecular weights reported by Gavine et al. (1984). However, Fischer-Colbrie et al. (1984) report an apparent molecular weight of 43000 for GpIII in the Laemmli gel system under reducing conditions; small variations in the gel system probably account for the differences in molecular weight.

3.5 Isolation of dopamine β -hydroxylase, glycoprotein III and glycoproteins J and K by electroeluting these glycoproteins from polyacrylamide gel slices

Glycoproteins eluted from LCL-agarose were separated in SDS polyacrylamide slab gels (6-15%) and slices containing DBH, GpJ, GpK and GpIII were cut from the gels. Glycoproteins were electroeluted from the gel slices as described in section 2.11. Figure 3.13 shows that electroeluted DBH, and glycoproteins III, J and K migrated as single bands on one-dimensional electrophoresis, and this together with two-dimensional electrophoresis of electroeluted DBH indicated that the glycoproteins were pure. Insufficient amount of glycoproteins J and K, and GpIII were electroeluted to be visible on two-dimensional gels stained with Coomassie Blue. After one-dimensional electrophoresis electroeluted DBH, and glycoproteins J and K were transferred to cellulose nitrate which was then decorated with Con A and horseradish peroxidase. The cellulose nitrate replica (fig. 3.8) showed single bands of electroeluted

glycoproteins J and K, indicating that the glycoproteins were pure and that GpJ did not appear to be contaminated while the DBH contained traces of higher Mr bands with GpK. These preparations were used for raising antibodies.

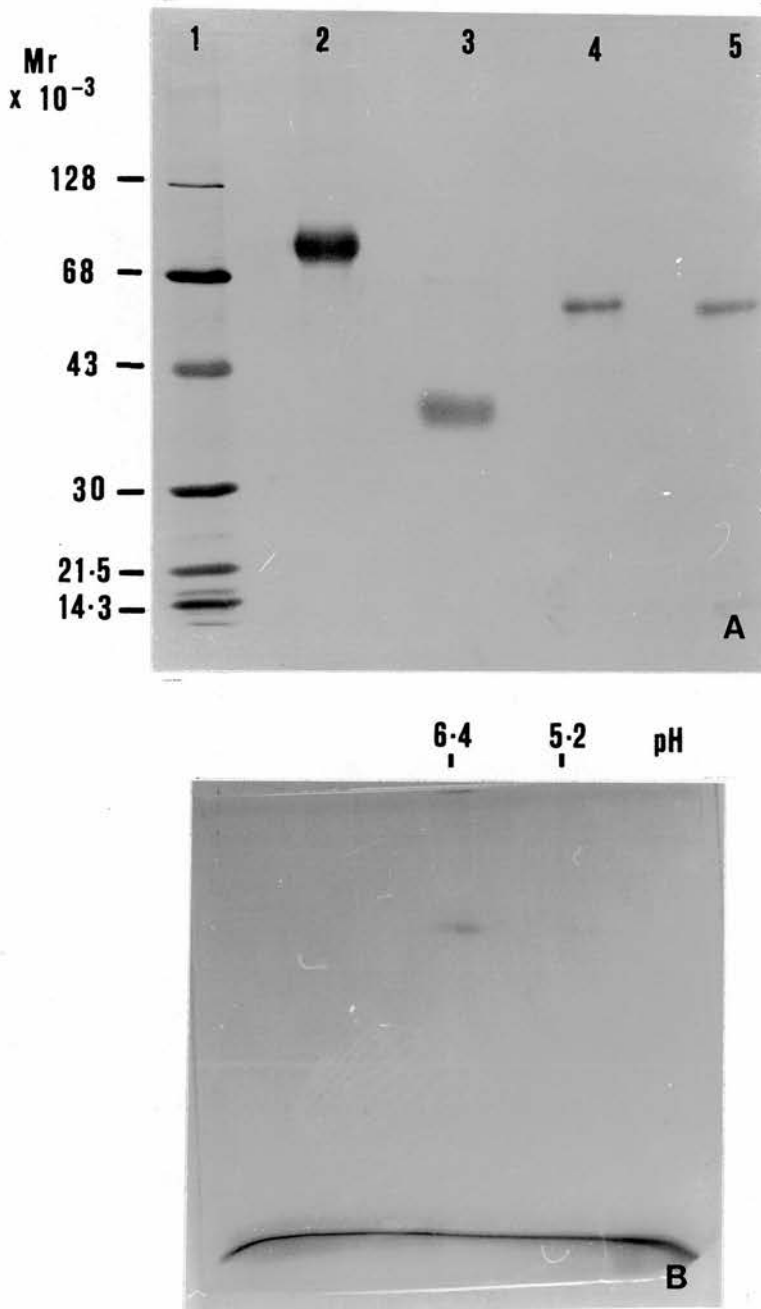


Figure 3.13 Electrophoretic analysis of electroeluted glycoproteins. The gels were stained with Coomassie Blue. (A) One-dimensional gel (6-15%). Track 1, standard proteins were as in section 2.15.4; track 2, electroeluted dopamine β -hydroxylase; track 3, electroeluted glycoprotein lll; track 4, electroeluted glycoprotein J; track 5, electroeluted glycoprotein K. (B) Two-dimensional gel of electroeluted dopamine β -hydroxylase.

CHAPTER FOUR
CHARACTERIZATION OF ANTISERA

4.1 Introduction

Glycoproteins J and K have no known enzymic activity and so specific antisera were required for studying them. Con A is a convenient ligand for glycoproteins J and K, but it is not specific because it binds to many other glycoproteins in chromaffin granules (Gavine et al., 1984). Membrane DBH, GpJ, GpK and Gplll were purified by chromatography on LCL-agarose and electroelution (described in sections 2.10, 2.11 and chapter 3) and guinea pigs and rabbits were immunized with the purified glycoproteins to produce polyclonal antisera. Cytochrome b-561 and Gplll have been unequivocally demonstrated to be chromaffin granule membrane components (Apps et al., 1980; Fischer-Colbrie et al., 1984), and antisera against these proteins can be used to identify the position of chromaffin granules and membranes in sucrose gradients, and hence to compare the locations of these proteins with those of glycoproteins J and K.

Antisera against DBH, Gplll and glycoproteins J and K were prepared as described in section 2.14. Rabbit antiserum against cytochrome b-561 was the gift of Dr. L. Kilpatrick. Antisera were diluted in 5% v/v heat-inactivated horse serum, 2.5% w/v BSA, 0.05% w/v NaN_3 , 20 mM Tris-HCl, 0.9% w/v NaCl, pH 7.2 (horse serum buffer in section 2.5) for characterization.

4.2 Antiserum against cytochrome b-561

Chromaffin granule membrane proteins were separated in a one-dimensional SDS polyacrylamide gel (8-15%) and proteins were transferred from individual tracks of the

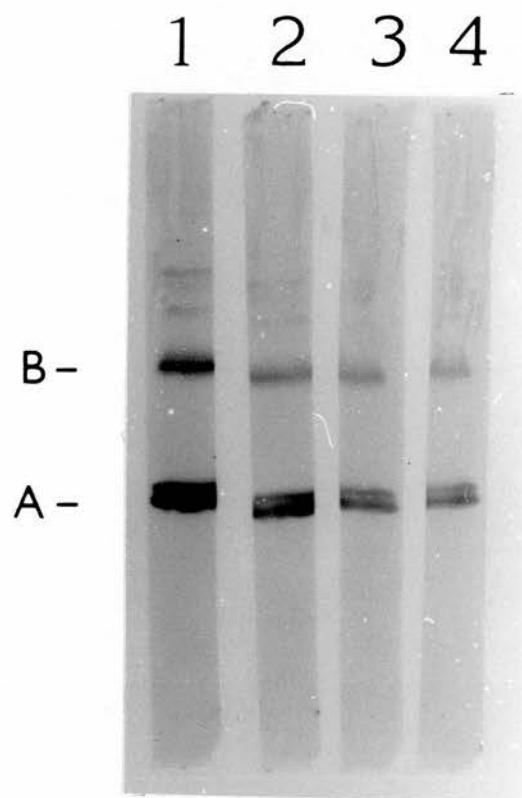


Figure 4.1 Autoradiograph of immune replicas of chromaffin granule membranes (30 μ g of protein/track) decorated with anti-cytochrome b-561 serum. Track 1, 100-fold dilution of antiserum; track 2, 200-fold dilution; track 3, 300-fold dilution; track 4, 400 fold dilution of antiserum. Band A is cytochrome b-561; B is thought to be undissociated cytochrome oligomer.

gelto strips of cellulose nitrate as described in section 2.5. The strips were decorated with various dilutions of anti-cytochrome b-561 serum. Figure 4.1 shows that the antiserum was specific for cytochrome b-561 because it labelled a doublet (band A, Mr 26000-28000). Another band (B) is thought to be undissociated cytochrome oligomer (Apps et al., 1980; Hunter et al., 1982). In subsequent experiments cellulose nitrate replicas were decorated with a 200-fold dilution of this antiserum.

4.3 Antiserum against membrane dopamine β -hydroxylase

Antiserum raised against electroeluted membrane DBH recognised both membrane and soluble DBH (fig. 4.2). Soluble DBH, purified by ion-exchange and lectin chromatography (Ljones et al., 1976), and soluble DBH deglycosylated by treatment with trifluoromethane sulphonic acid (Stewart et al., 1984), were the gift of Dr. D. K. Apps. As shown in figure 4.2 not only purified soluble DBH, but deglycosylated soluble DBH was labelled by this anti-membrane DBH serum, the deglycosylated protein migrating with an apparent Mr of 72000. Similar results were obtained by Apps et al. (1985). The identity of the bands of lower Mr in tracks 3 and 5 of figure 4.2A is unknown but they may be proteolysis products of soluble DBH. The bands and spots indicated by arrows in figure 4.2 probably correspond to glycoproteins J and K because they migrated with the same Mr and pI. These glycoproteins were eluted from LCL-agarose together with DBH. Overloading of preparative SDS polyacrylamide

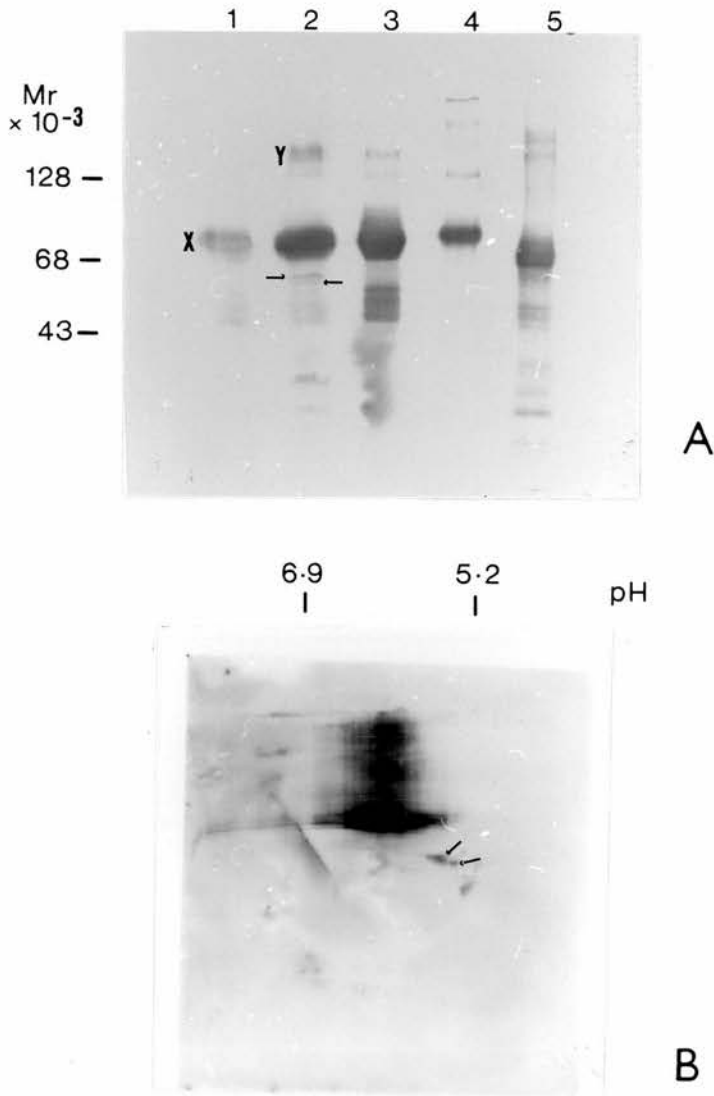


Figure 4.2 Autoradiographs of immune replicas decorated with anti membrane dopamine β -hydroxylase serum (diluted 100-fold). (A) Replica of a one-dimensional gel (6-15%). Track 1, chromaffin granule lysate (150 μ g); track 2, chromaffin granule membranes (150 μ g); track 3, purified soluble dopamine β -hydroxylase (40 μ g); track 4, purified membrane dopamine β -hydroxylase; track 5, deglycosylated soluble dopamine β -hydroxylase (40 μ g). (B) Replica of a two-dimensional gel of chromaffin granule membranes (300 μ g). The bands and spots indicated by the arrows are probably glycoproteins J and K. Band X is the DBH monomer (M_r 75000) and Y is its dimer.

slab gels with LCL-eluate (section 2.11) may result in small amounts of GpJ and GpK migrating together with DBH and these glycoproteins would then be electroeluted with DBH. However, electroeluted DBH did not appear to be contaminated with GpJ and GpK (chapter 3) and glycoproteins J and K were not visible when a gel of electroeluted membrane DBH was decorated with the antiserum (track 4, fig. 4.2).

4.4 Antisera against glycoprotein lll

Antisera were raised against Gplll, purified from chromaffin granule membranes, in a rabbit and in a guinea pig. Autoradiographs of cellulose nitrate replicas decorated with guinea pig serum are shown in figure 4.3. The antiserum was specific for Gplll and the pre-immune serum did not react with any membrane component. The bands (M_r 64000 - 66000) on the replica of the two-dimensional gel (indicated by an arrow in fig. 4.3C) may be keratin and are discussed in section 4.6. The antiserum also labelled soluble Gplll after transfer of chromaffin granule lysate proteins to cellulose nitrate (autoradiograph not shown). Some of this glycoprotein, like DBH, has previously been reported to be located in the matrix (Fischer-Colbrie *et al.*, 1984).

Immune replicas of chromaffin granule membranes were decorated with rabbit antiserum and are shown in figure 4.4. Band Y (M_r 75000) in figure 4.4 is discussed in section 4.6 and presumably results from non-specific binding of antibodies to a chromaffin granule membrane component.

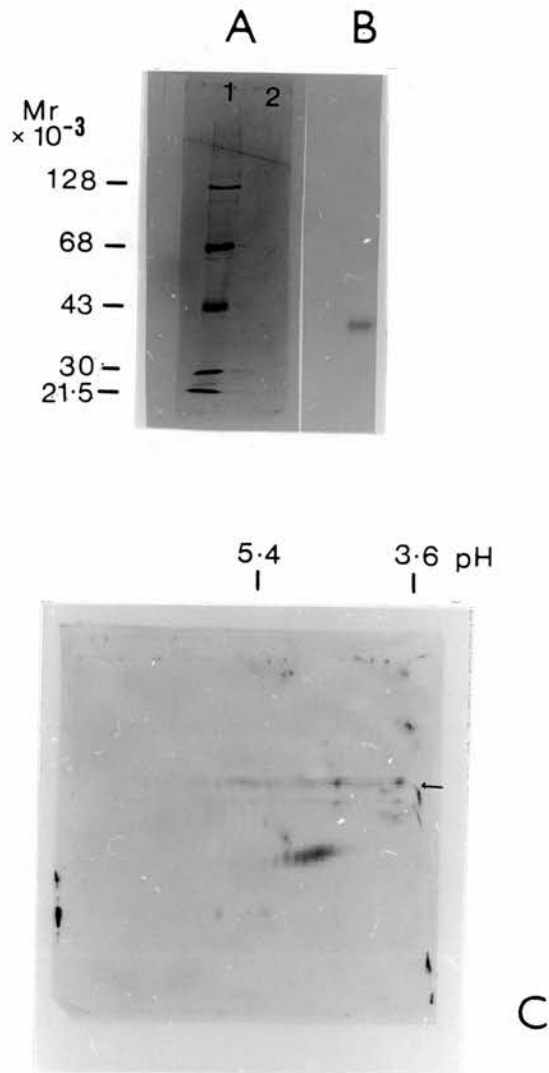


Figure 4.3 Autoradiographs of cellulose nitrate replicas decorated with a guinea pig serum against glycoprotein 111 (diluted 100-fold). (A) Replica of a 6-10% gel decorated with pre-immune serum. Track 1, ¹⁴C-labelled standard proteins were as in section 2.15. Track 2, chromaffin granule membranes (100 µg). (B) Replica of a 6-15% gel of membranes (100 µg) decorated with antiserum. (C) Two-dimensional gel of membranes (300 µg) decorated with antiserum. The bands indicated by the arrow may be keratin.

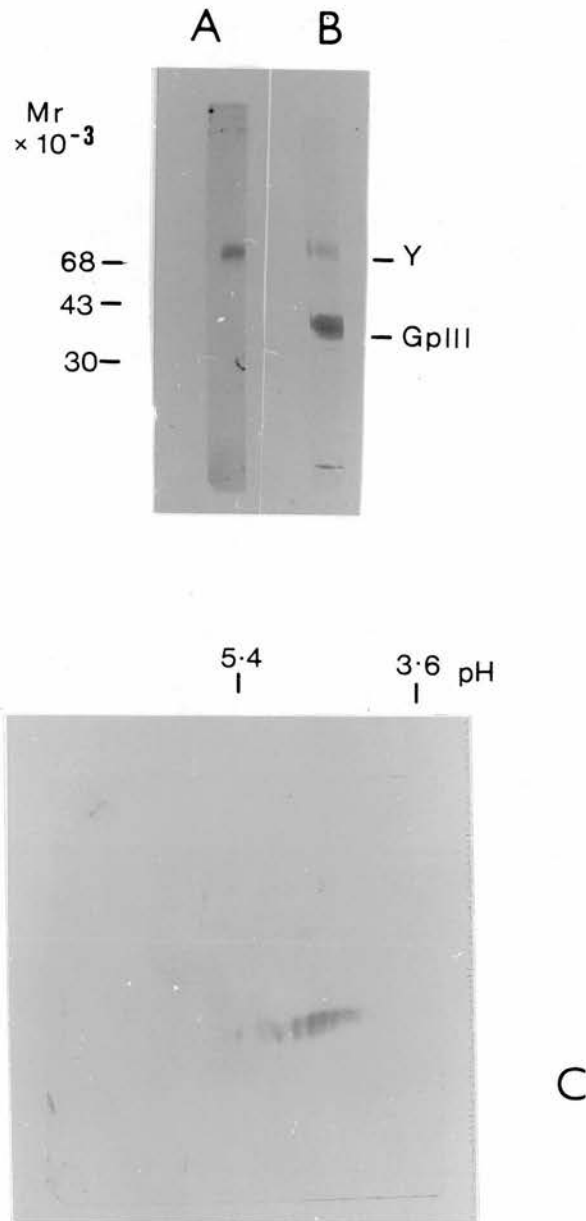


Figure 4.4. Autoradiographs of cellulose nitrate replicas decorated with rabbit anti-glycoprotein lll serum (A) One-dimensional gel (6-15%) of chromaffin granule membranes (100 µg) decorated with pre-immune serum (diluted 100-fold). (B) One-dimensional gel (6-15%) of membranes (150 µg) decorated with antiserum (diluted 100-fold). (C) Two-dimensional gel of membranes (300 µg) decorated with antiserum (diluted 50-fold). The identity of band Y is not clear. Abbreviation; GpIII, glycoprotein lll

The guinea pig antiserum was used in subsequent experiments.

4.5 Antisera against glycoproteins J and K

Electroeluted GpJ and GpK were injected into separate guinea pigs, but antisera produced by the two animals recognized both glycoproteins. Figure 4.5 shows autoradiographs of replicas decorated with antiserum from the guinea pig injected with GpJ. The pre-immune serum appeared to recognize a band (X in fig. 4.5; Mr 50000) that does not correspond to either GpJ or GpK; presumably this was a result of non-specific binding. None of the chromaffin granule lysate proteins was labelled by anti-glycoproteins J and K serum after transfer to cellulose nitrate (see fig. 4.5B) indicating that GpJ and GpK, unlike DBH and Gplll, are not present in the matrix of chromaffin granules. Some major matrix proteins, such as chromogranin A, are seen as faint negative images in this track. Track 2 (fig. 4.5B) shows that the antiserum recognizes GpK in addition to GpJ.

An antiserum, which recognized glycoproteins J and K, was also produced by the guinea pig immunized with GpK (Fig. 4.6). The identity of band Z (Mr 75000) in Figure 4.6 is discussed in section 4.6.

There seem to be two possible explanations for the production of a mixed anti-glycoproteins J and K serum by both guinea pigs: (1) electroeluted GpJ used for immunization may have been contaminated with GpK, and vice versa; or (2) glycoproteins J and K may be related proteins and so have common antigenic determinants. One

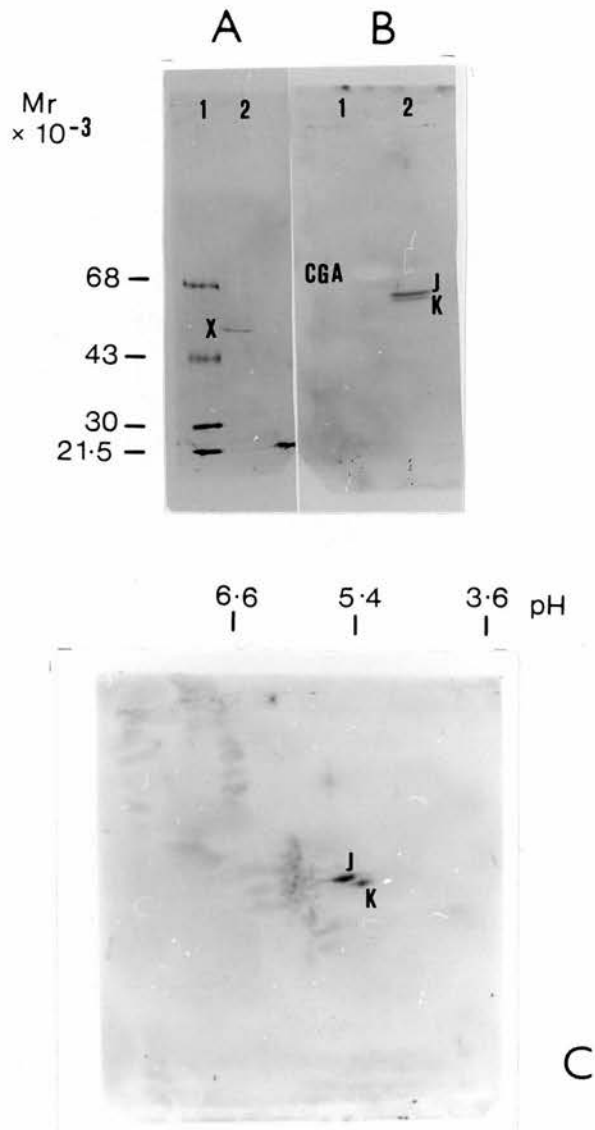


Figure 4.5 Autoradiographs of replicas decorated with anti-glycoproteins J and K serum (diluted 100-fold) raised in a guinea pig injected with glycoprotein J. (A) Replica of a one-dimensional gel (6-10%) decorated with pre-immune serum. Track 1, ^{14}C -labelled standard proteins were as in section 2.15. Track 2, chromaffin granule membranes (80 μg). (B) One-dimensional gel (6-15%) decorated with antiserum. Track 1, chromaffin granule lysate (100 μg); track 2, membranes (80 μg). (C) Two-dimensional gel of membranes (300 μg) decorated with antiserum. The identity of band X is not known. The negative image of chromogranin A (CGA) is indicated.

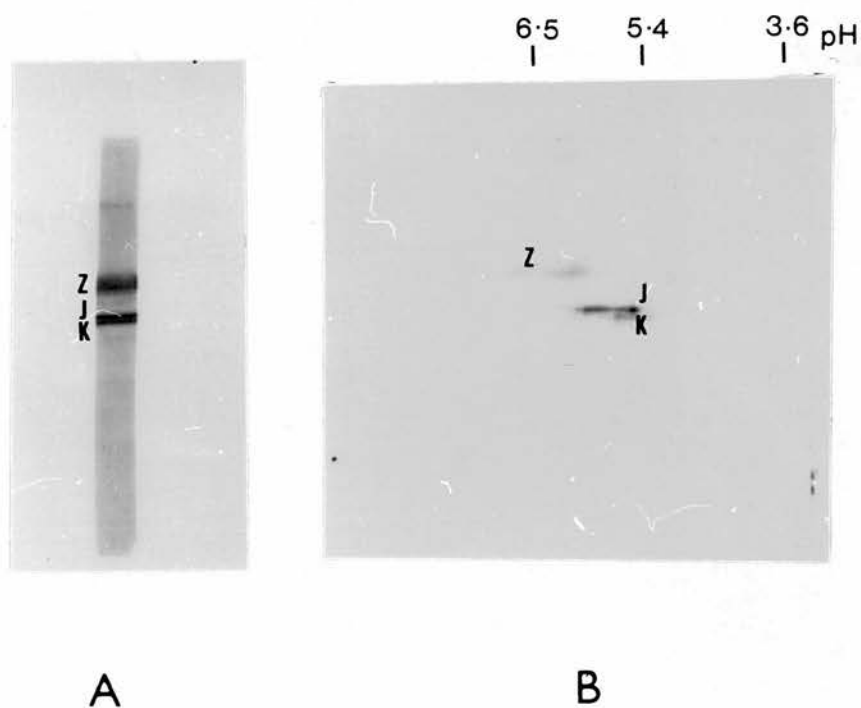


Figure 4.6 Autoradiographs of replicas decorated with anti-glycoproteins J and K serum (diluted 100-fold) raised in a guinea pig injected with glycoprotein K. (A) One-dimensional gel (6-15%) of chromaffin granule membranes (120 μ g). (B) Two-dimensional gel of membranes (300 μ g). The identity of band Z is not clear.

dimensional SDS gels stained with Coomassie Blue and Con A replicas of GpJ and GpK, as purified by electroelution and as used for immunization, failed to show that either was contaminated with the other (chapter 3). However, small amounts of contaminating protein that were not detected could be present. Attempts to separate GpJ and GpK by FPLC on an anion exchange column (Mono Q) were unsuccessful, probably because of extensive aggregation of these membrane proteins on the column. I was also unable to raise antisera against these two glycoproteins in rabbits. The question of whether GpJ and GpK are related is discussed later in chapter 8.

The antiserum produced by the guinea pig immunized with GpJ was used in subsequent experiments.

4.6 Non-specific labelling of components by the antisera

Two bands (Mr 64000-66000) were visible on some autoradiographs of immune replicas and an example is shown Figure 4.3. The appearance of these bands has been reported by Ochs (1983) and they probably correspond to keratin subunits (Mr 65000-68000). Contamination was reduced as the concentration of 2-mercaptoethanol in the sample loading buffer was reduced from 5% v/v to 0.5% v/v presumably because fewer disulphide bonds in the keratin molecules were broken and more keratin therefore, remained at the top of the gel.

A band (Mr 75000; band Y in Fig. 4.4 and band Z in Fig. 4.6) was visible in autoradiographs of replicas decorated with antisera against Gplll and glycoproteins J and K, and with a pre-immune serum from a rabbit.

This component was poorly focussed in two-dimensional electrophoresis (fig. 4.6B) and migrated with a very similar pI and apparent Mr to those of DBH. Labelling of band Y (fig. 4.4) by the pre-immune serum might simply reflect non-specific binding of antibodies to the most abundant protein in the chromaffin granule membrane; i.e. DBH. Also, labelling of this component decreased with successive bleeds of the guinea pigs immunized with Gp111 and GpJ (figs. 4.3B, 4.5B).

Despite non-specific binding of antibodies in the sera to various components, all these antisera were suitable for investigating the distribution of glycoproteins in sucrose gradients and for the investigation of membrane topography. This was because bands on autoradiographs corresponding to GpJ, GpK and Gp111 were well separated from the contaminants, and therefore easily identified, while other bands could be ignored.

CHAPTER FIVE

SUBCELLULAR LOCATION OF GLYCOPROTEIN J AND
GLYCOPROTEIN K IN THE ADRENAL MEDULLA

5.1 Introduction

Glycoproteins J and K are major Con A - binding proteins. It has been suggested that they are confined to chromaffin granules in the adrenal medulla because they are not visible on Con A - overlays of mitochondrial or microsomal fractions from this tissue (Gavine et al., 1984). However, Con A is not very specific because it labels many other chromaffin granule membrane proteins. A much better method for studying the subcellular location of GpJ and GpK is to follow their distribution in sucrose density gradients of chromaffin granules and membranes, using antisera against these proteins. Cytochrome b-561 and Gplll have been shown unequivocally to be chromaffin granule components (Apps et al., 1980; Fischer-Colbrie et al., 1984), so they can be used as markers for the membranes of these organelles. Glycoproteins J and K should be found in the same fractions from a sucrose gradient as cytochrome b-561 and Gplll if they are also confined to chromaffin granules in the adrenal medulla.

This approach was used to decorate cellulose nitrate replicas of gels of gradient fractions with antisera followed by ^{125}I -labelled protein A. Bands on autoradiographs of the replicas corresponding to GpJ, GpK, Gplll and cytochrome b-561 were then quantified by densitometry. The heights or areas of the densitometer peaks were used as a measure of the amount of each of the proteins in each fraction. In preliminary experiments a linear relationship between increasing binding of ^{125}I -labelled protein A

Table 5.1 Simple linear regression analysis of the measurements in figures 5.1-5.4

peak height or area versus protein (fig)	slope \pm standard error (cm/ μ g or cm ² / μ g)	intercept \pm standard error (cm or cm ²)
GpJ height (5.1A)	0.148 \pm 0.019**	1.378 \pm 0.560*
GpJ area (5.1B)	0.085 \pm 0.014**	0.380 \pm 0.428
GpK height (5.2A)	0.124 \pm 0.016**	-0.056 \pm 0.481
GpK area (5.2B)	0.070 \pm 0.013**	-0.280 \pm 0.382
Gp111 height (5.3A)	0.203 \pm 0.028**	4.834 \pm 0.869**
Gp111 area (5.3B)	0.211 \pm 0.027**	2.069 \pm 0.838*
b-561 height (5.4A)	0.338 \pm 0.036**	0.906 \pm 0.589
b-561 area (5.4B)	0.082 \pm 0.014**	-0.098 \pm 0.237

'Student t' test: ** = significantly different from zero $P < 0.01$; * = significantly different from zero $P < 0.05$.

to antigen-antibody complexes and membrane protein loaded onto the gel was demonstrated.

5.2 Quantitation by the immune replica technique

The linear relationship between binding of ^{125}I -labelled protein A to antigen-antibody complexes and the amount of chromaffin granule membrane protein loaded onto a single track of the gel was established using the immune replica technique. Quantitation by this method has been discussed by Vaessen et al., 1981 and Riezman (1984). Different quantities of chromaffin granule membranes (2-30 μg or 5-50 μg of protein) were electrophoresed in the same SDS polyacrylamide gel, transferred to cellulose nitrate sheets, then the sheets were decorated with antisera against glycoproteins J and K, Gp111 and cytochrome b-561. The antibody-antigen complexes were visualized using ^{125}I -labelled protein A and autoradiographed. Autoradiographs were cut into slices corresponding to individual tracks of the gel and the slices were scanned in a densitometer (described in section 2.15.3).

The relationships between both peak height and peak area corresponding to the GpJ, GpK, Gp111 and cytochrome b-561 bands and the amount of membrane protein loaded onto each track of the gel are shown in figures 5.1-5.4. A simple linear regression analysis was performed on each set of measurements (see table 5.1) and the regression equations are given in the legends to figures 5.1-5.4. The computer programme for the regression analysis was

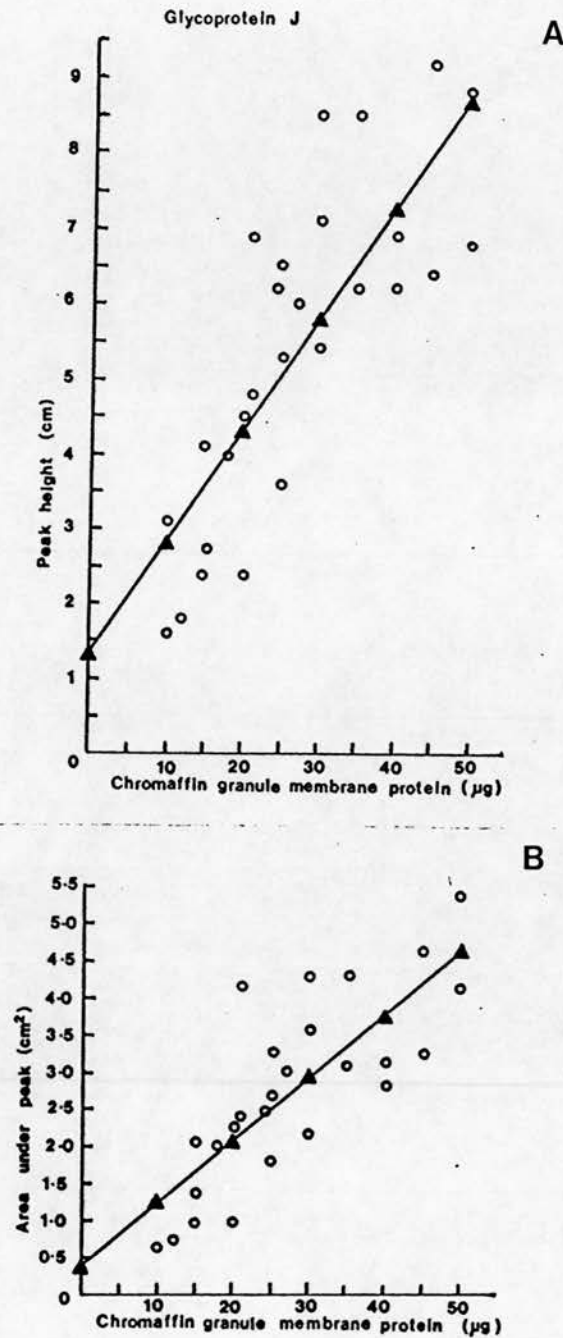


Figure 5.1 ^{125}I -labelled protein A bound to glycoprotein J-antibody complexes. \circ indicates measurements from densitometric scans of three autoradiographs. The points on the straight lines (\blacktriangle) were calculated from the simple linear regression equations : (A) $y = 0.148x + 1.378$ (B) $y = 0.085x + 0.380$. 1 cm = 0.04 absorbance units at 625 nm.

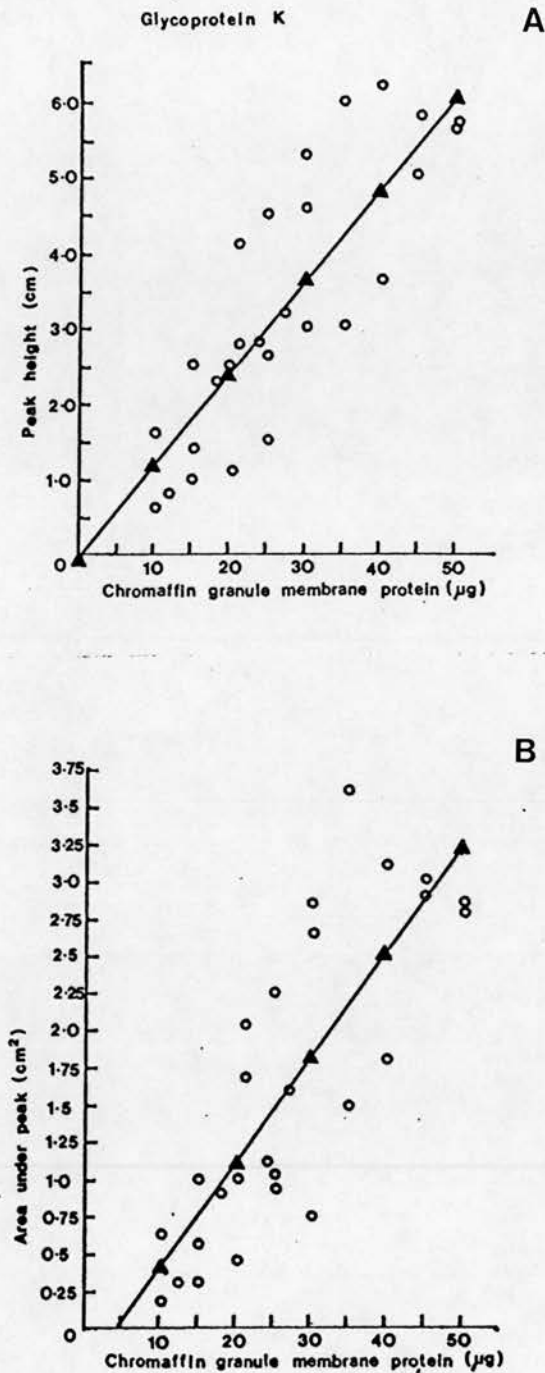


Figure 5.2 ^{125}I -labelled protein A bound to glycoprotein K - antibody complexes. ○ indicates measurements from densitometric scans of three autoradiographs. The points on the straight lines (▲) were calculated from the simple linear regression equations : (A) $y = 0.124x - 0.056$; (B) $y = 0.070x - 0.280$. 1 cm = 0.04 absorbance units at 625 nm.

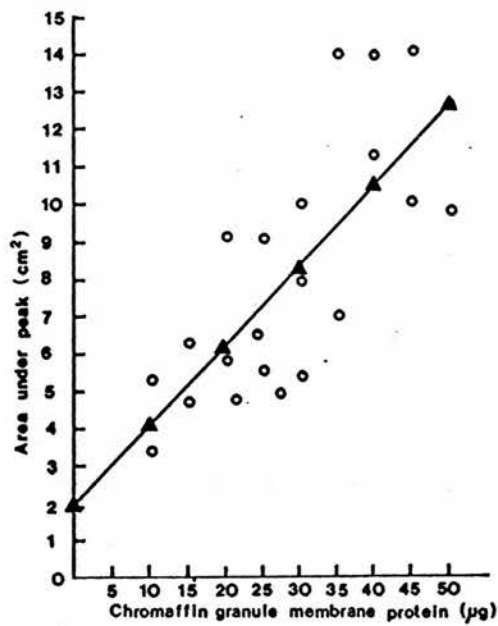
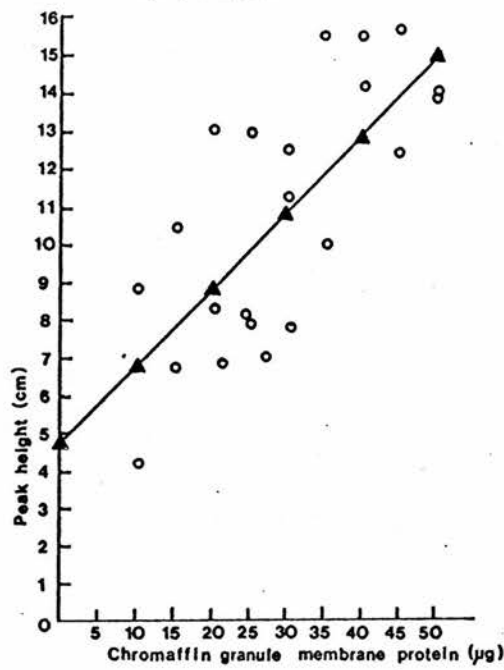


Figure 5.3 ^{125}I -labelled protein A bound to glycoprotein III - antibody complexes. ○ indicates measurements from densitometric scans of three autoradiographs. The points on the straight lines (▲) were calculated from the simple linear regression equations : (A) $y = 0.203x + 4.834$; (B) $y = 0.211x + 2.069$. 1 cm = 0.045 absorbance units at 625 nm.

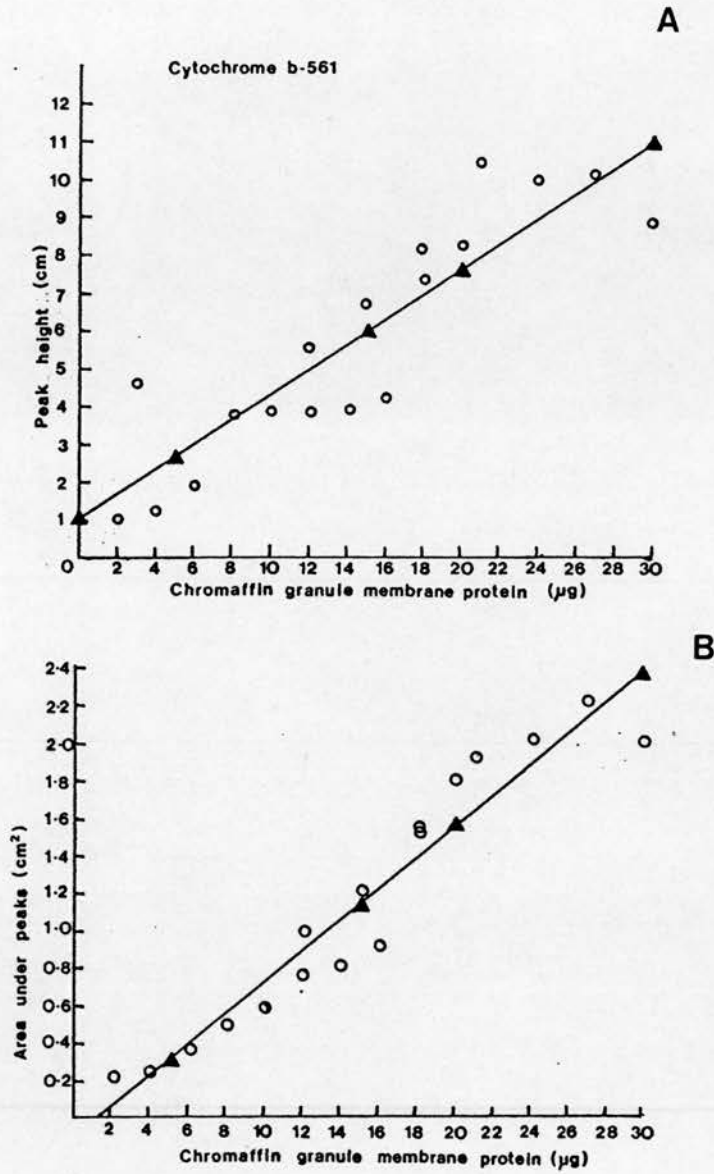


Figure 5.4 ¹²⁵I-labelled protein A bound to cytochrome b-561 - antibody complexes. ○ indicates measurements from densitometric scans of two autoradiographs. The points on the straight lines (▲) were calculated from the simple linear regression equations : (A) $y = 0.338x + 0.906$; (B) $y = 0.082x - 0.098$. 1 cm = 0.075 absorbance units at 625 nm.

written by Dr. I. Nimmo (Biochemistry Department). The amount of ^{125}I -labelled protein A bound to antigen-antibody complexes increased linearly up to 50 μg of membrane protein loaded onto a single track of the gel (GpJ, GpK and Gpllll; figs. 5.1-5.3 and table 5.1) or up to 30 μg of protein (cytochrome b-561; fig. 5.4 and table 5.1). In most cases the intercept on the y axis was not significantly different from zero (table 5.1) but the lines in figs. 5.1A and 5.3 do not pass through the origin. This is because it was difficult to estimate accurately the background resulting from ^{125}I -labelled protein A binding non-specifically to the replica, and no correction was made for this effect. Difficulty in estimating the background probably accounts also for the scatter of the data in figs. 5.1-5.4.

Plots of peak height versus area under the same peak were linear for GpJ, GpK and Gpllll, indicating a good correlation between these two parameters. So, only the heights of the peaks were measured in subsequent experiments. Cytochrome b-561 migrates as a doublet in polyacrylamide gels (Apps et al., 1980), and so two peaks were visible on the densitometric scans (fig. 5.6). The area under the peaks of cytochrome b-561 was therefore a more accurate measure of the amount of this protein in a fraction.

5.3 Distribution of cytochrome b-561, glycoprotein lll, glycoproteins J and K in a sucrose density gradient of purified chromaffin granule membranes

Pure chromaffin granule membranes were prepared

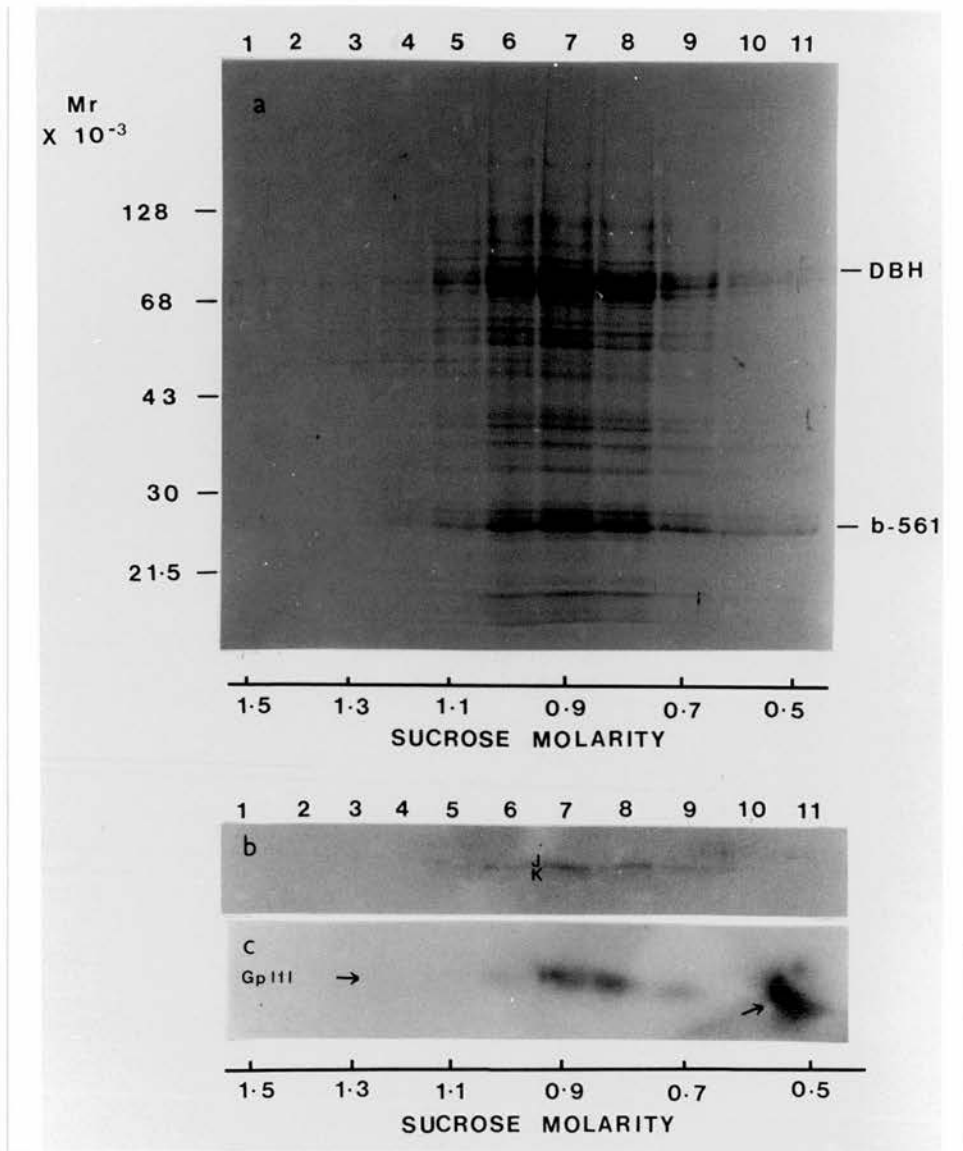


Figure 5.5(a) One-dimensional gel (6-15%), stained with Coomassie Blue, of fractions collected after centrifugation of purified chromaffin granule membranes in a gradient of 0.4 - 1.6 M sucrose. (b and c) Autoradiographs of replicas of the fractions decorated with (b) anti-glycoproteins J and K serum, (c) anti-glycoprotein lll serum. The mark indicated by the arrow in (c) is an artefact resulting from uneven contact of the cellulose nitrate sheet with the gel during transfer of proteins. Abbreviations: b-561, cytochrome b-561; GpIII, glycoprotein lll; DBH, dopamine β -hydroxylase.

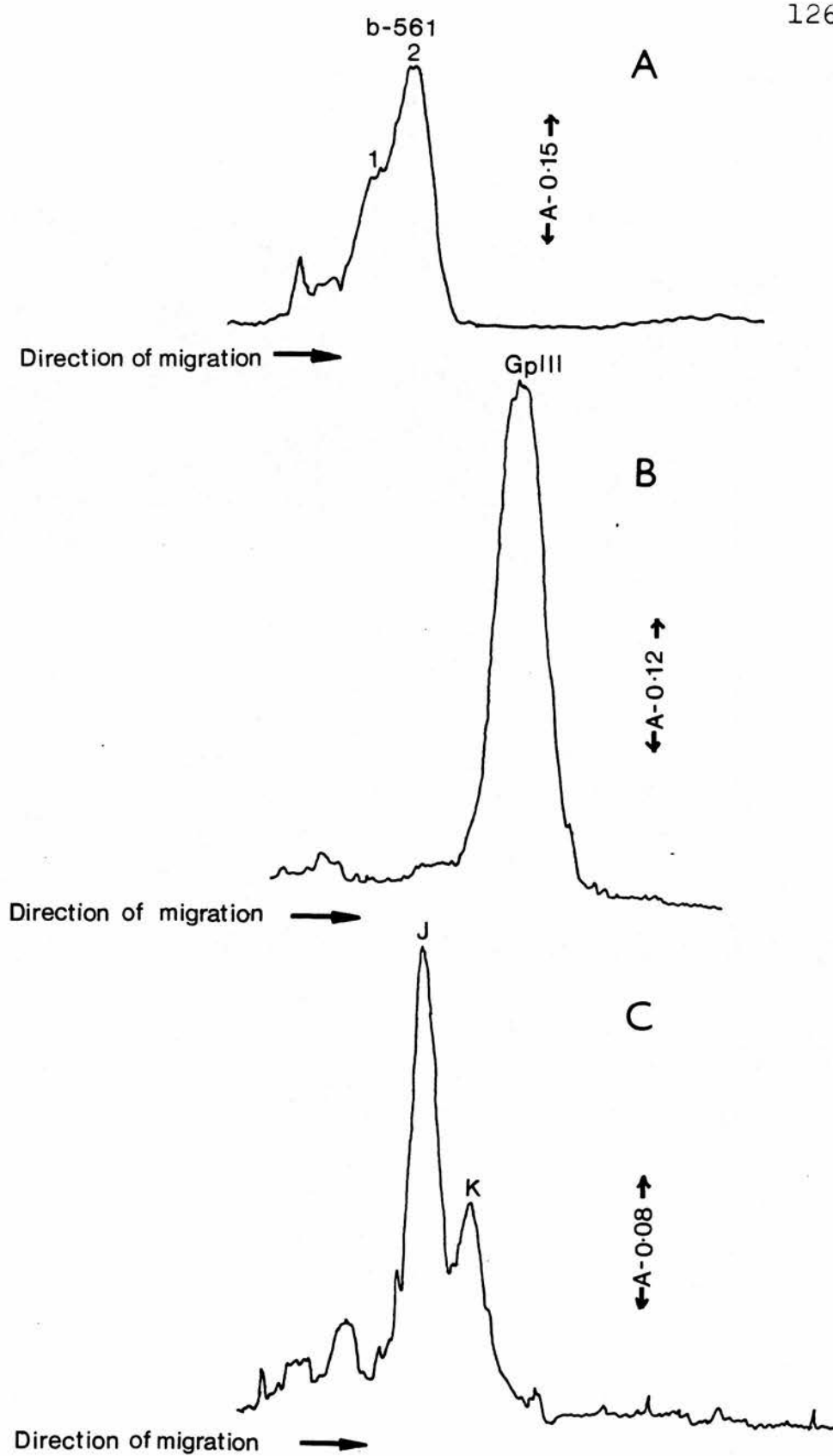
(section 2.2) and layered under linear gradients of 0.4-1.6 M sucrose as described in section 2.8. The gradients were centrifuged to equilibrium, fractionated, then 25 μ g of protein from the peak fraction and the same volume of each of the other fractions were electrophoresed in a polyacrylamide gel. The amount of protein loaded from each fraction was thus within the linear response range of the immune replica technique.

A Coomassie Blue stained gel of fractions from a gradient is shown in figure 5.5a. The chromaffin granule membrane proteins DBH and cytochrome b-561 are clearly visible in tracks 6, 7 and 8 (fractions containing 0.95, 0.85 and 0.77 M sucrose); only a small amount of protein sedimented at the bottom (tracks 1, 2 and 3) and at the top (track 11) of the gradient. Nevertheless, cytochrome b-561 and DBH are visible in track 11 of fig. 5.5a.

Proteins were transferred from gels of gradient fractions to cellulose nitrate sheets and the replicas were decorated with specific antisera. Autoradiographs of replicas decorated with anti-glycoproteins J and K serum and anti-Gplll serum are illustrated in figure 5.5 (b and c). Bands of GpJ, GpK and Gplll can be seen in tracks 6, 7, 8 and 9 and these fractions were also rich in DBH and cytochrome b-561 (fig. 5.5a).

Autoradiographs of replicas of gradient fractions were cut into slices corresponding to individual tracks of the gel and the slices were scanned. Traces from replicas of the peak gradient fraction (containing 0.85 M sucrose)

Figure 5.6 Densitometric scans of autoradiograph slices. Proteins in the fraction containing 0.85 M sucrose from a density gradient of purified chromaffin granule membranes were separated by gel electrophoresis and transferred to cellulose nitrate sheets. The replicas were decorated with : (A) anti-cytochrome b-561 serum; (B) anti-glycoprotein lll serum; (C) anti-glycoproteins J and K serum, and autoradiographed. Only sections of the scans containing peaks are shown and the two peaks of cytochrome b-561 are indicated. Absorbance was measured at 625 nm. Abbreviations are as in fig. 5.5.

**Figure 5-6**

are shown in figure 5.6; the peaks of GpJ and GpK were easily distinguished. The band of GpJ on autoradiographs (fig. 5.5b) was always more intense than the band of GpK; the ratio of peak heights (GpJ : GpK) was approximately 2.5 : 1. The antiserum was raised by a guinea pig immunized with GpJ, and so this ratio of peak heights could simply reflect a higher titre of antibodies against GpJ in the serum.

The distribution of GpJ and GpK in a sucrose density gradient of purified membranes is shown in figure 5.7. Both glycoproteins followed the distribution of Gplll and of total protein, but the distribution of cytochrome b-561 was slightly different. Cytochrome b-561 and the other proteins were most abundant in the fraction containing 0.85 M sucrose. GpJ, GpK and Gplll were not found in the fraction containing 1.05 M sucrose (fig. 5.7) but cytochrome b-561 was present in this fraction. A possible explanation for the wide distribution of cytochrome b-561 in the gradient is that antibodies against the mature protein (which is not glycosylated) are able to recognize those proteins molecules that are located in more dense membranes such as the endoplasmic reticulum and the Golgi apparatus, while antibodies directed against the glycoproteins are unable to recognize precursor forms. Cytochrome b-561 was also found at the top of the gradient (fig. 5.7 and track 11, fig. 5.5a) and so the abundance of this protein in the granule membrane might also explain its wide distribution in the gradient.

Figure 5.7 Distribution of proteins in a density gradient (0.4 - 1.6 M sucrose) of purified chromaffin granule membranes. Replicas of gradient fractions were decorated with antisera and autoradiographs of replicas were cut into slices. The slices were scanned and heights of peaks of glycoprotein J, glycoprotein K and glycoprotein lll, or area under the peaks of cytochrome b-561 were measured. (○), total protein in each fraction; (Δ), cytochrome b-561; (□), glycoprotein J; (▲), glycoprotein K; (●), glycoprotein lll. Sucrose molarities were calculated from refractive index measurements.

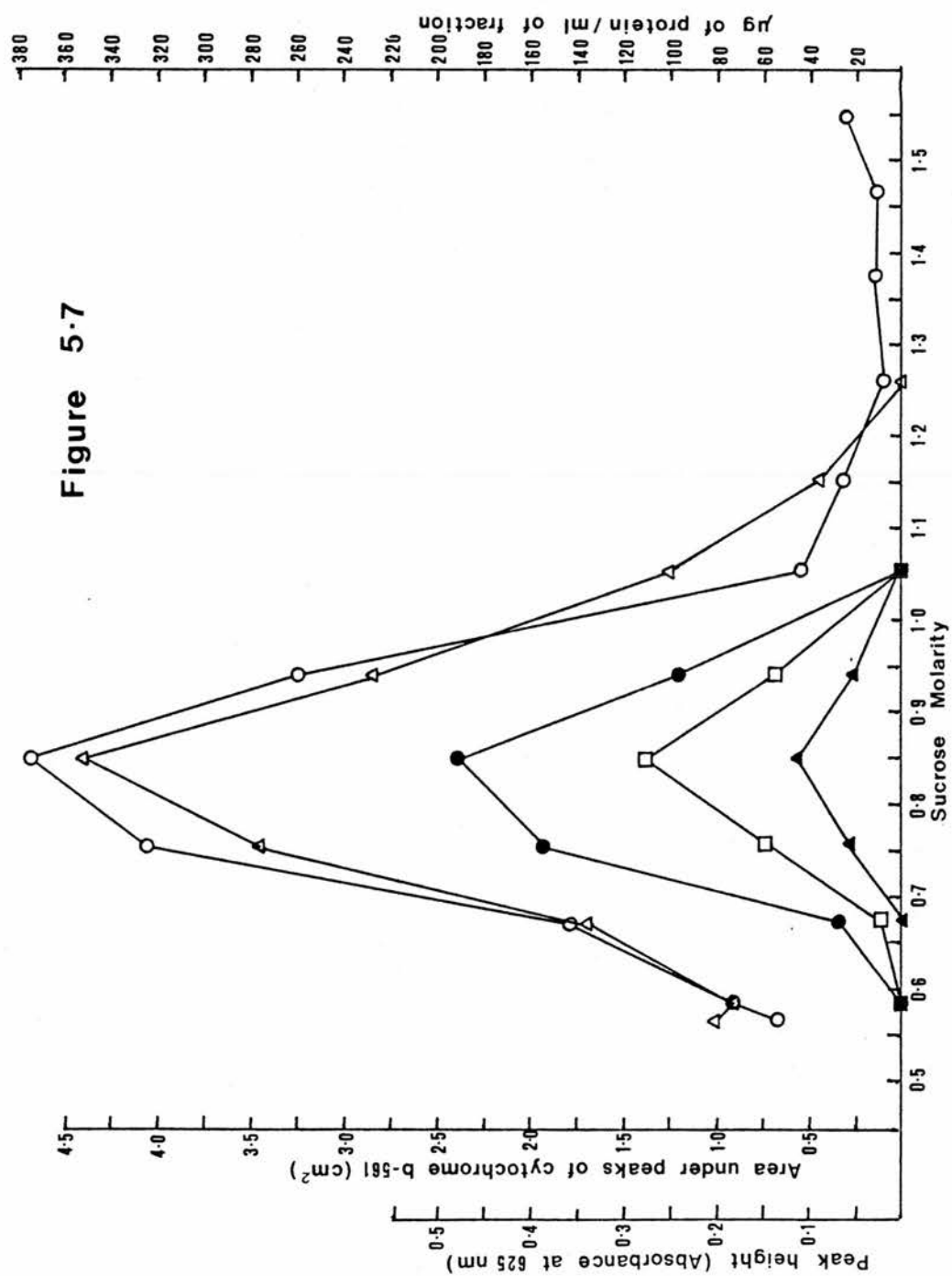


Figure 5-7

In summary, the greatest amount of GpJ and GpK was found in the fraction containing 0.85 M sucrose together with Gplll and cytochrome b-561, showing that GpJ and GpK are specific components of the chromaffin granule membrane.

5.4 Distribution of cytochrome b-561, glycoprotein lll, and glycoproteins J and K in a sucrose density gradient of crude chromaffin granules

Chromaffin granules, mitochondria, lysosomes and microsomes (crude chromaffin granules in section 2.2) were centrifuged in a linear gradient of 0.5 - 2.0 M sucrose, a 'cushion' of 2.5 M sucrose underneath the gradient preventing the granules from pelleting. The first fraction from the gradient contained 2.2 - 2.3 M sucrose because the 'cushion' was mixed with 2.0 M sucrose from the bottom of the gradient during fractionation.

The distribution of GpJ, GpK, Gplll, cytochrome b-561 and mitochondrial F_1 ATPase in a gradient was determined by decorating replicas of electrophoresed gradient fractions with antisera against the proteins. All the fractions from one gradient were electrophoresed in the same polyacrylamide gel; 100 μ g of protein from the peak fraction and the same volume of the other fractions were loaded onto the gel. Because membrane proteins comprise only 20% of the total granule protein (Winkler and Carmichael, 1982), it was assumed that the linear relationship between protein and increasing amount

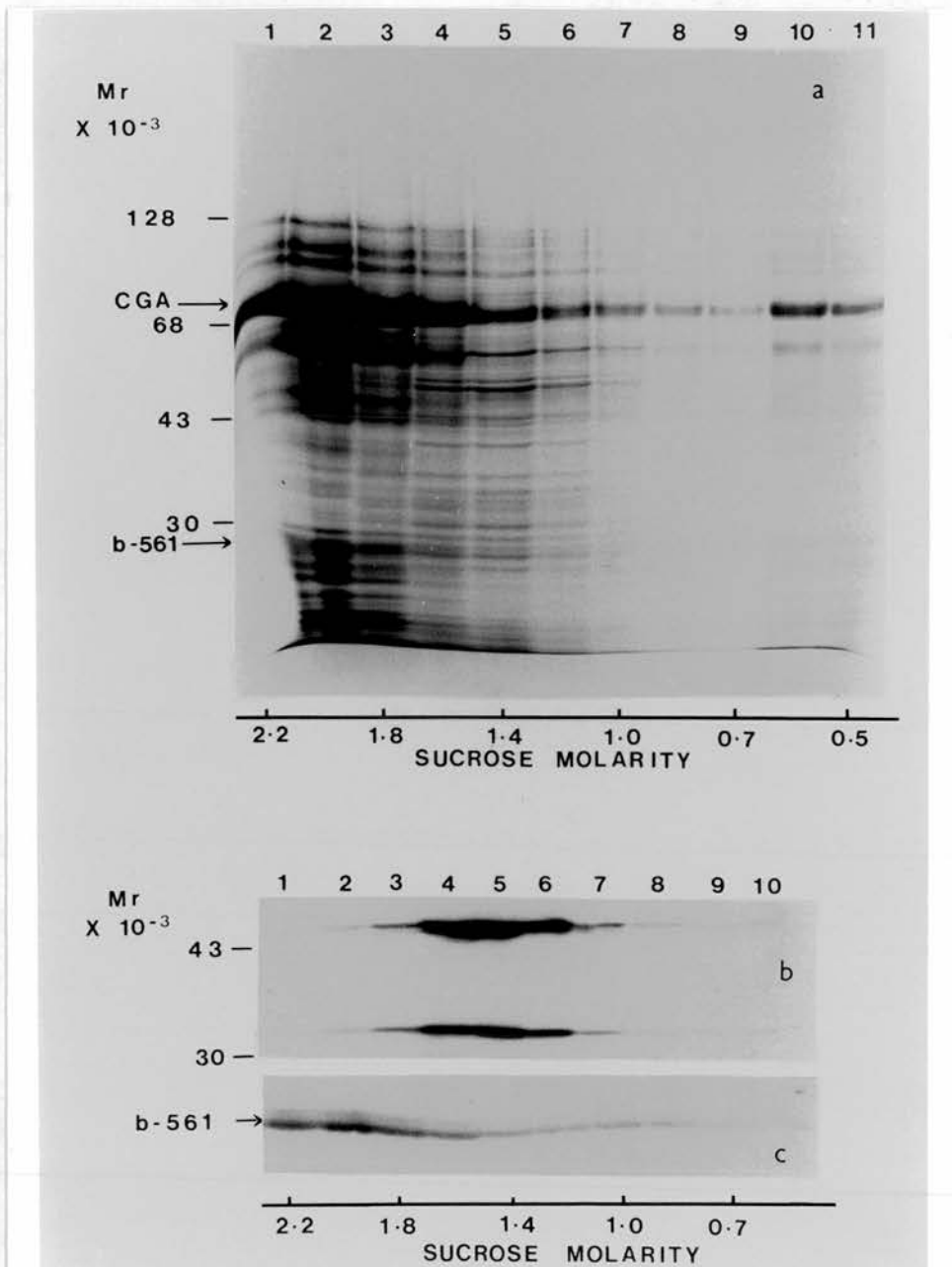


Figure 5.8(a) One-dimensional gel (6-15%), stained with Coomassie Blue, of fractions collected after centrifugation of crude chromaffin granules on a linear gradient of 0.5 - 2.0 M sucrose. The gradient was layered over a 'cushion' of 2.5 M sucrose, so the first fraction (2.2 M sucrose) contained a mixture of 2.5 M sucrose and 2.0 M sucrose from the bottom of the gradient. (b and c) Autoradiographs of replicas of fractions decorated with (b) anti-mitochondrial F₁ ATPase serum, and (c) anti-cytochrome b-561 serum. Abbreviations: CGA, chromogranin A; b-561, cytochrome b-561.

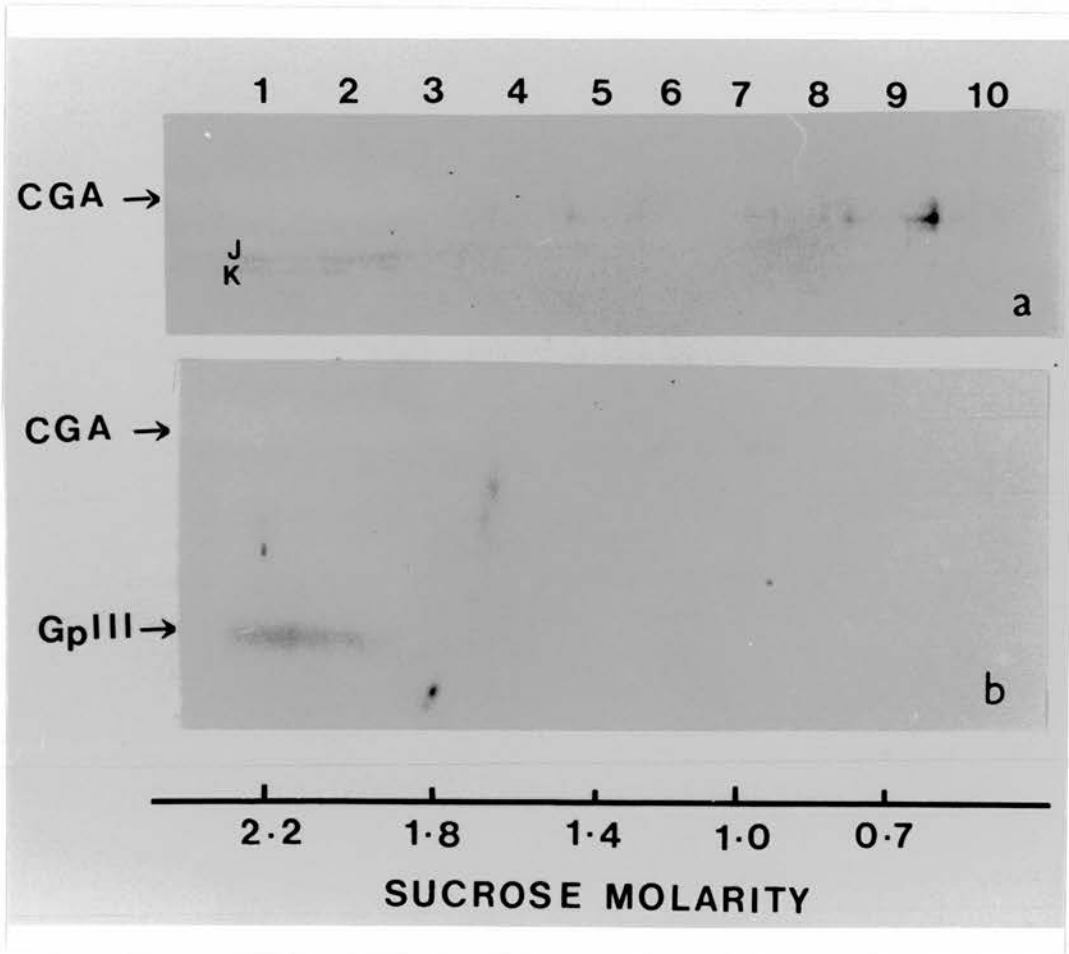


Figure 5.9 Autoradiographs of replicas of fractions from a sucrose density gradient of crude chromaffin granules (as in fig. 5.8) decorated with (a) anti-glycoproteins J and K serum; (b) anti-glycoprotein 111 serum. Negative bands of chromogranin A are indicated. Abbreviations: CGA, chromogranin A; Gp111, glycoprotein 111.

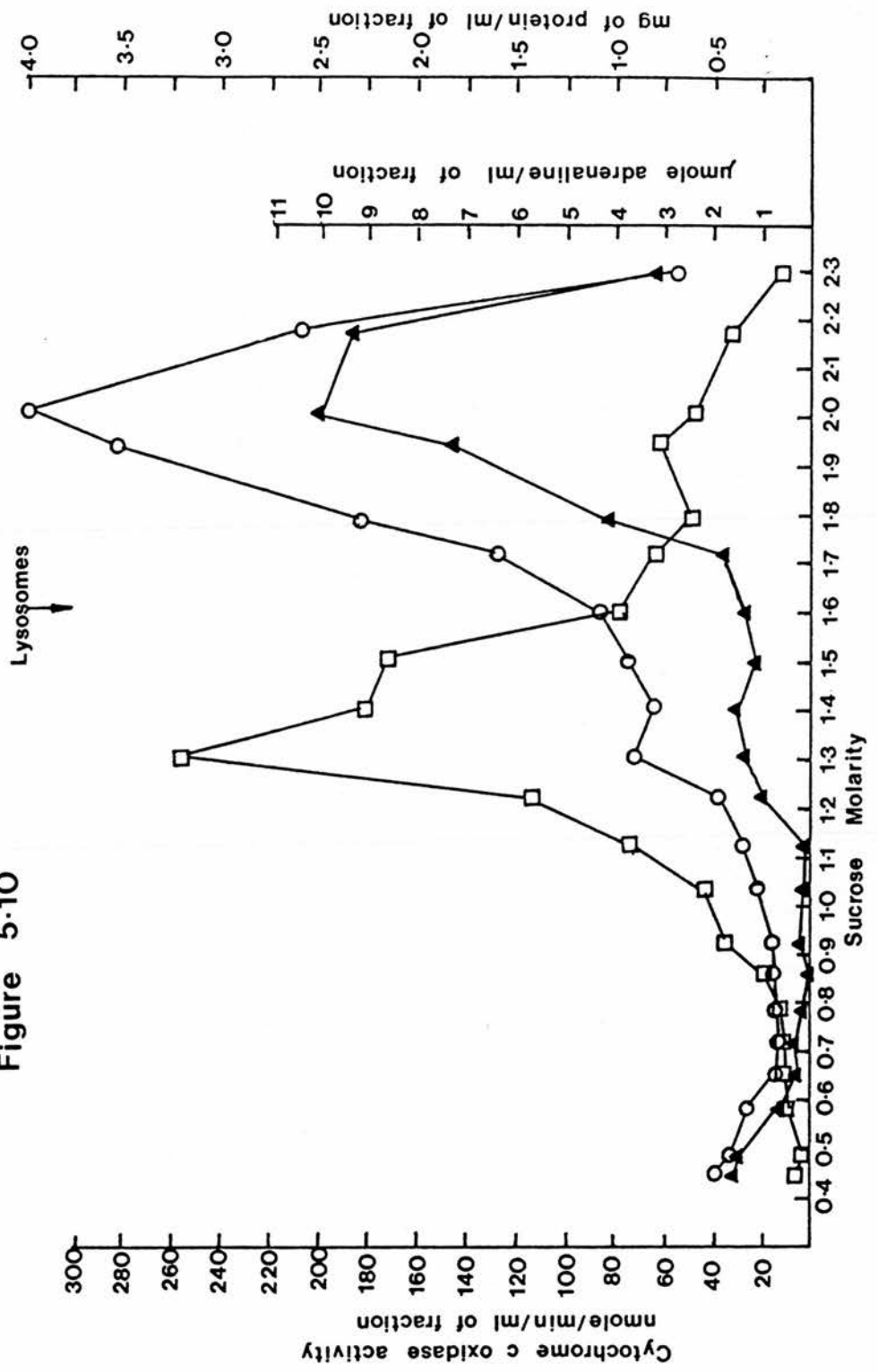
of ^{125}I -labelled protein A bound to antigen - antibody complexes would apply under these conditions (section 5.2). Autoradiographs of immune replicas of fractions from a gradient were scanned and heights of peaks corresponding to GpJ, GpK, Gp111 and cytochrome b-561 were used as a measure of the quantity of the proteins in the fractions.

Figure 5.8a shows a gel, stained with Coomassie Blue, of fractions from a sucrose gradient of crude chromaffin granules. The fraction containing 2 M sucrose is rich in chromogranin A and cytochrome b-561 (track 2). The sharply focussed bands in tracks 4, 5 and 6 of figure 5.8 (1.2-1.6 M sucrose) presumably correspond to mitochondrial proteins including F_1 ATPase. Autoradiographs of immune replicas of the fractions are shown in figures 5.8 and 5.9; the two sucrose gradients used for these figures were not identical. Gp111, GpJ and GpK were found in fractions containing 2.2 and 2.0 M sucrose (tracks 1, 2 in fig. 5.9). Chromogranin A was not labelled by antisera against glycoproteins J and K and Gp111 (chapter 4) and, because of its abundance, appears as a white negative band in tracks 1 and 2 of figure 5.9. Cytochrome b-561 was present in all the fractions (fig. 5.8c); the α , β and γ subunits of F_1 ATPase were found mostly in fractions containing 1.2-1.6 M sucrose (tracks 4, 5 and 6 in fig. 5.8b).

Cytochrome c oxidase (Mason et al., 1973) and the α , β and γ subunits of F_1 ATPase (Apps et al., 1983) were used as markers for adrenal mitochondria. The position of chromaffin granules in gradients was

Figure 5.10 Activity of cytochrome oxidase and the adrenaline concentration in fractions collected after centrifugation of crude chromaffin granules in a density gradient of 0.5 - 2.0 M sucrose (as in fig. 5.8). (●), total protein in each fraction; (▲) adrenaline concentration; (□), cytochrome c oxidase activity. Lysosomes equilibrate at about 1.6 M sucrose. Sucrose molarities were calculated from refractive index measurements.

Figure 5.10



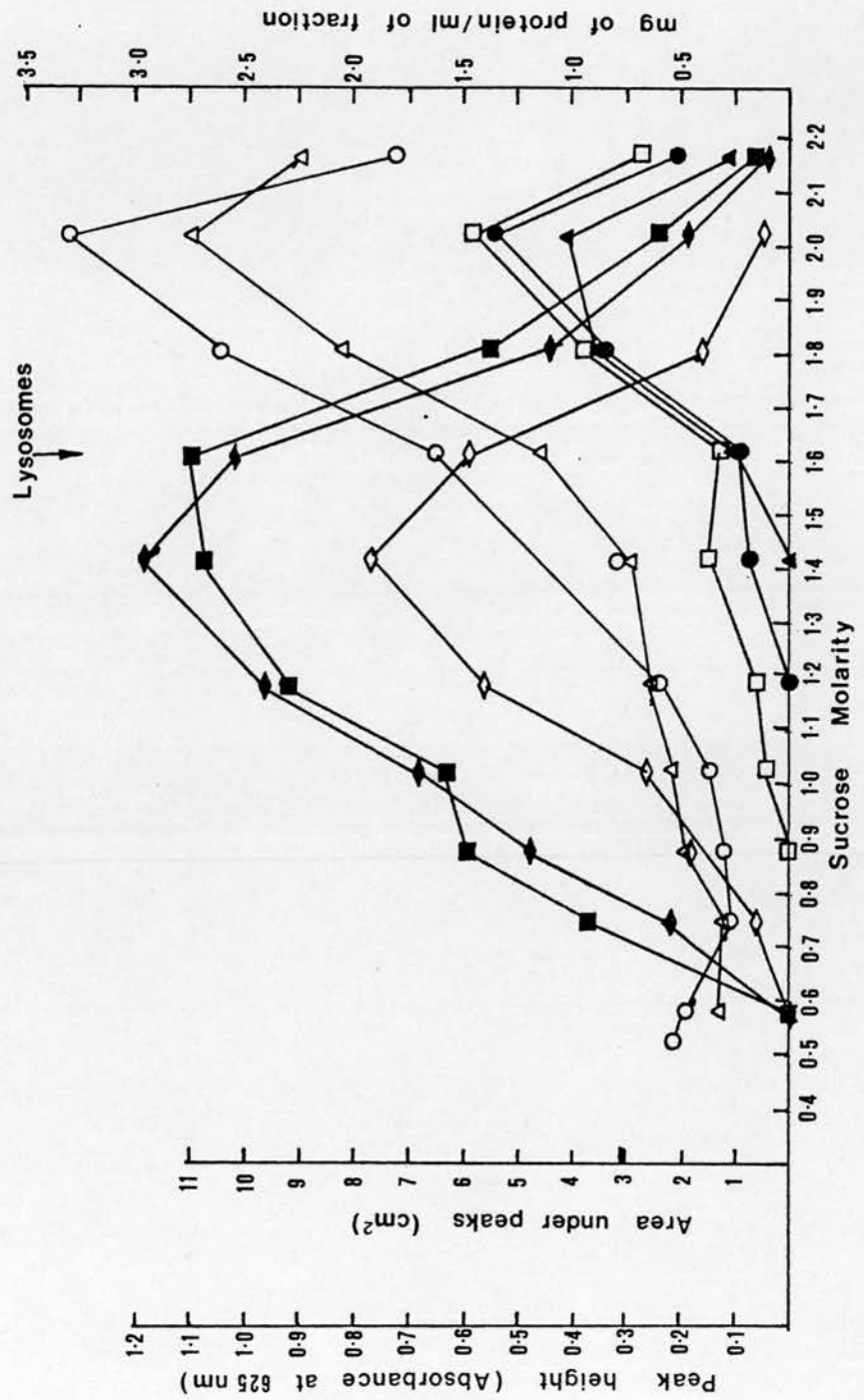
determined by measuring the adrenaline concentration and the amounts of Gplll and cytochrome b-561 in each fraction. The distribution of cytochrome c oxidase and adrenaline in a gradient is shown in figure 5.10 and that of F_1 ATPase, cytochrome b-561 and Gplll in figure 5.11; the two gradients used for these figures were not the same.

In figure 5.11 it can be seen that the distribution of the α subunit of F_1 ATPase does not precisely follow the β and γ subunits. Nevertheless, the maximum activity of cytochrome c oxidase was found at 1.3 M sucrose (fig. 5.10), the β and γ subunits of F_1 sedimented to equilibrium at 1.4 M sucrose (fig. 5.11), so mitochondria equilibrated at 1.3-1.4 M sucrose. Granules sedimented to equilibrium at 2 M sucrose where the highest concentration of adrenaline (fig. 5.10) and the greatest amount of cytochrome b-561 and Gplll (fig. 5.11) were found. Lysosomes are relatively heterogeneous; other workers have found that they equilibrate at about 1.6 M sucrose in gradients of adrenal medullary homogenates (Smith and Winkler, 1966; Laduron and Belpaire, 1968; Schneider, 1970).

The distribution of GpJ and GpK in the gradient follows that of Gplll and total protein (fig. 5.11); the greatest amount of glycoproteins J and K was found in the fraction containing chromaffin granules. Traces of GpJ and GpK were also found in the region of the gradient containing mitochondria (1.4 M sucrose, fig. 5.11), but much more of the glycoproteins equilibrated

Figure 5.11 Distribution of proteins after centrifugation of crude chromaffin granules in a gradient of 0.5 - 2.0 M sucrose, (as in fig. 5.8). (○), total protein in each fraction; (Δ), cytochrome b-561; (□), glycoprotein J; (●), glycoprotein K; (▲), glycoprotein III; (■), α subunit of F_1 ATPase; (◊), β subunit of F_1 ATPase; (◈), γ subunit of F_1 ATPase. Lysosomes equilibrate at about 1.6 M sucrose. Sucrose molarities were calculated from refractive index measurements.

Figure 5.11



with granules at 2 M sucrose. The spread of these glycoproteins is attributed to granule heterogeneity. On the other hand, cytochrome b-561 was again found to have an anomalously wide distribution in the gradient (fig. 5.8c and 5.11). This may result from the high abundance of cytochrome b-561 in the granule membrane, or alternatively (as mentioned in section 5.3) antibodies in the serum may recognize the protein in subcellular fractions other than mature granules.

The small increase in the amount of protein, cytochrome b-561 and adrenaline at the top of the gradient (0.5-0.65 M sucrose) is assumed to result from broken membranes sedimenting in this region (figs. 5.10 and 5.11). The ratio of peak heights (GpJ : GpK) was approximately 1.4 : 1, somewhat lower than the ratio of 2.5 : 1 calculated in section 5.3 from gradients of purified membranes. However, the same anti-glycoproteins J and K serum was used to decorate replicas of gels of fractions from gradients of purified membranes and crude granules. The difference in the ratios cannot be explained by soluble forms of GpJ and GpK occurring in the matrix because the same antiserum did not label soluble lysate proteins after transfer to cellulose nitrate.

In conclusion, the region of the gradient (2.0 M sucrose) containing the most protein, Gplll, cytochrome b-561 and adrenaline was also rich in GpJ and GpK. Only small amounts of GpJ and GpK were found in fractions containing lysosomes and mitochondria. These results

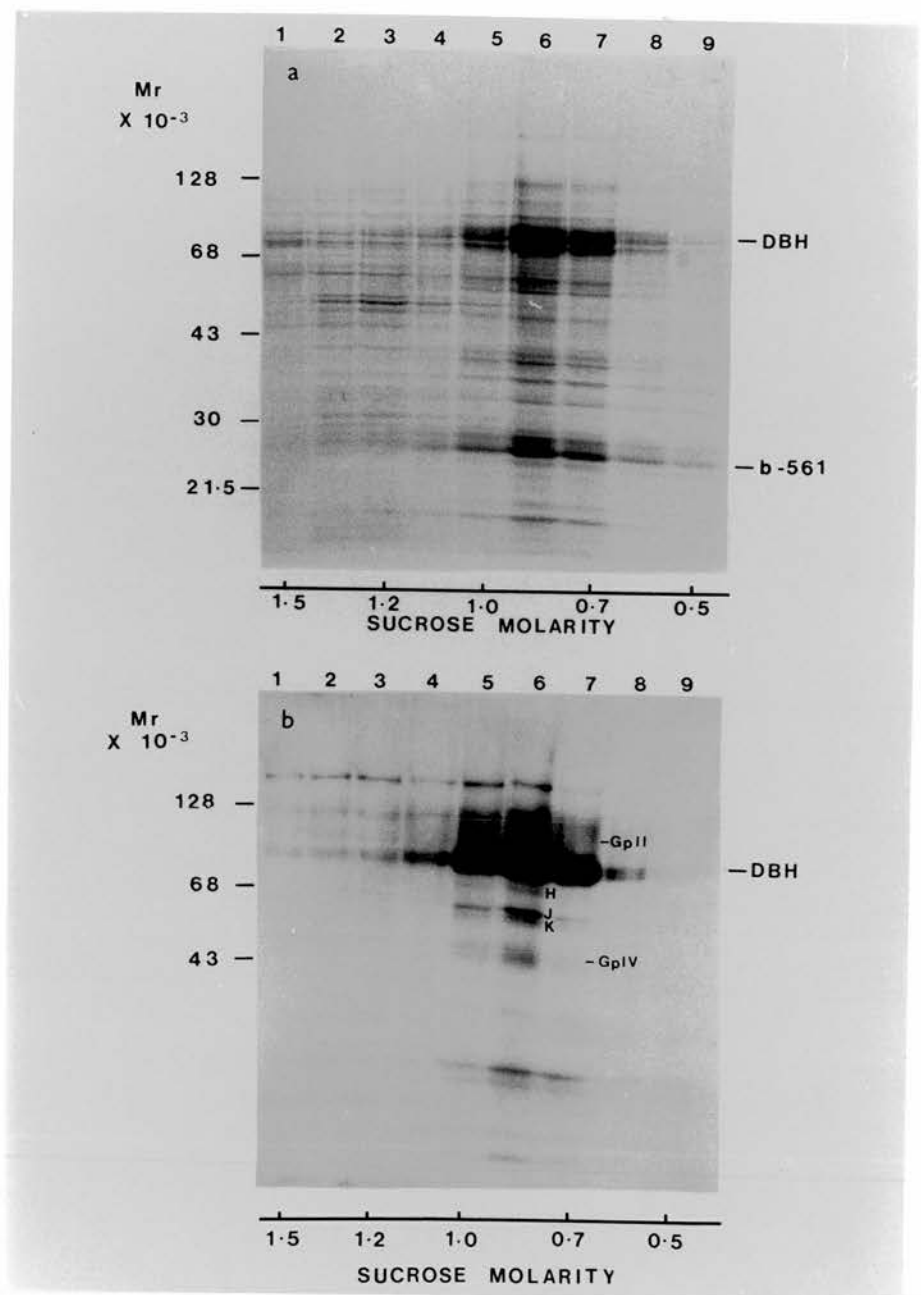


Figure 5.12 One-dimensional gels (6-15%) of fractions from a density gradient (0.4 - 1.6 M sucrose) of crude chromaffin granule membranes. (a) Stained with Coomassie Blue. (b) Replica decorated with ¹²⁵I-labelled concanavalin A. Abbreviations: DBH, dopamine β -hydroxylase; GpII, glycoprotein II; GpIV, glycoprotein IV; b-561, cytochrome b-561.

strongly suggest that GpJ and GpK are specific components of chromaffin granules in the adrenal medulla.

5.5 Distribution of glycoproteins J and K in a sucrose density gradient of crude membranes

Chromaffin granule membranes contaminated with adrenal mitochondria and other adrenal membranes (crude membranes in section 2.2) were centrifuged to equilibrium in a gradient of 0.4 - 1.6 M sucrose. Fractions from the gradient were electrophoresed in a polyacrylamide gel and then proteins were transferred to cellulose nitrate sheets. Decoration of the replicas with ^{125}I -labelled Con A was followed by autoradiography to visualize the labelled glycoproteins.

A Coomassie Blue - stained gel of fractions from a gradient is illustrated in fig. 5.12a. DBH and cytochrome b-561 were stained heavily by Coomassie Blue in track 6 of figure 5.12a and this fraction (0.83 M sucrose) also contained the most protein (results not shown). So, membranes sedimented to equilibrium at 0.83 M sucrose and this compares favourably with the gradient of purified membranes (section 5.3) in which membranes equilibrated at 0.85 M sucrose (fig. 5.7). The sharply focussed bands in tracks 2 and 3 of fig. 5.12a (1.4-1.25 M sucrose), presumably contain mitochondrial proteins.

A replica of the gradient fractions decorated with Con A is shown in figure 5.12b. Strong labelling of glycoproteins H, J, K, Gp111, GpIV and DBH can be seen, with a peak in track 6, the region of the gradient containing chromaffin granule membranes (0.83 M sucrose).

In fig. 5.12b, the Con A - binding protein migrating with an apparent Mr of 150000 is unlikely to be the dimer of DBH because the dimer migrates as an indistinct and poorly focussed band. Mitochondrial proteins were labelled poorly by Con A (tracks 2, 3), similar results being reported by Gavine et al. (1984), and glycoproteins H, J and K cannot be seen in these tracks.

These results therefore provide further confirmation that GpJ and GpK are confined to chromaffin granules in the adrenal medulla. Also, although no antibody was available, fig. 5.12b suggests that GpH may also be a specific component of chromaffin granules.

CHAPTER SIX
MEMBRANE TOPOGRAPHY. LOCALIZATION OF
GLYCOPROTEINS IN BOVINE BRAIN AND IN
ISOLATED CHROMAFFIN CELLS

6.1 Introduction

Early studies of membrane topography indicated that chromomembrin B (identical with cytochrome b-561; Apps et al., 1980) is exposed on the cytoplasmic surface of chromaffin granules (König et al., 1976) whereas DBH faces the matrix (Kirshner, 1962; König et al., 1976). Intact chromaffin granules and broken membranes have been digested with pronase or labelled with the following impermeant reagents: lactoperoxidase/Na¹²⁵I as a reagent for tyrosine residues and N-(iodoacetyl aminoethyl) -5-naphthylamine -1-sulphonic acid as a reagent for cysteine residues (Abbs and Phillips, 1980). The results of these experiments indicated that cytochrome b-561 is a transmembrane protein, whereas DBH is exposed only on the inner (matrix) surface of the granule membrane. The organization of cytochrome b-561 in the membrane has been investigated by immunological techniques, using a specific antiserum against this protein (Hunter et al., 1982). Cytochrome b-561 migrates on SDS polyacrylamide gels as a doublet, apparent Mr 26000-28000 (Apps et al., 1980) and is degraded to a polypeptide of apparent Mr 22000-25000 when intact granules are incubated with pronase. This digestion results in a loss of antibody binding, showing that the antiserum against cytochrome b-561 reacts with antigenic determinants on the cytoplasmic side of the granule membrane (Hunter et al., 1982).

Complement fixation experiments with anti-glycoprotein lll showed that this antiserum reacted strongly with broken membranes but only very weakly with intact granules, indicating that the antigenic determinants of glycoprotein lll, together with those of DBH, are exposed on the matrix surface of the membrane (Hunter et al., 1982; Fischer-Colbrie et al., 1984).

Most of the evidence in the literature is consistent with the carbohydrate portions of chromaffin granule membrane glycoproteins being on the inner (extracytoplasmic) surface of the membrane. Intact granules are not labelled by galactose oxidase / KB^3H_4 or by Con A but broken membranes are labelled by these reagents (Eagles et al., 1975; Huber et al., 1979; Abbs and Phillips, 1980). Meyer and Burger (1976) suggested that there were receptors for WGA on the cytoplasmic surface of the granule membrane, but this has not been confirmed.

A number of chromaffin granule proteins have been found in tissues other than the adrenal medulla. In view of the recent demonstration of Gplll, a protein found both in the granule matrix and in the membrane, in adeno- and neurohypophysis (Fischer-Colbrie et al., 1984), an attempt was made to demonstrate that glycoproteins J and K are found in tissues from brain. In addition, the location of DBH, Gplll and glycoproteins J and K in isolated chromaffin cells was studied by immunofluorescence microscopy.

6.2 Topography of proteins within the chromaffin granule membrane

Preliminary experiments were carried out to establish the conditions for digesting proteins on the cytoplasmic surface of intact granules with proteases and for preparing membranes from digested granules for SDS polyacrylamide gel electrophoresis. Cytochrome b-561 and DBH are major bands which are easily identified on gels, stained with Coomassie Blue. A decrease in apparent Mr of cytochrome b-561 was an indication that proteolysis had occurred on the cytoplasmic side of the granule membrane. DBH is not accessible to proteases in intact granules; its presence on gels was therefore used as an indication that the proteases were being adequately inactivated during sample preparation.

Crude chromaffin granules (15 mg of protein/ml) were incubated with trypsin, chymotrypsin, pronase or papain (100 µg/ml) for 18 h at room temperature with gentle agitation. Then, benzamidine (final concentration 1 mM) and PMSF (final concentration 50 µM) were added to granules treated with chymotrypsin, trypsin or pronase. Iodoacetamide (final concentration 2 mM) was added to those treated with papain. The granules were washed by centrifugation in buffered sucrose containing the protease inhibitors and then granules were centrifuged through 1.6 M sucrose (section 2.2) to remove those which had lysed during incubation with proteases.

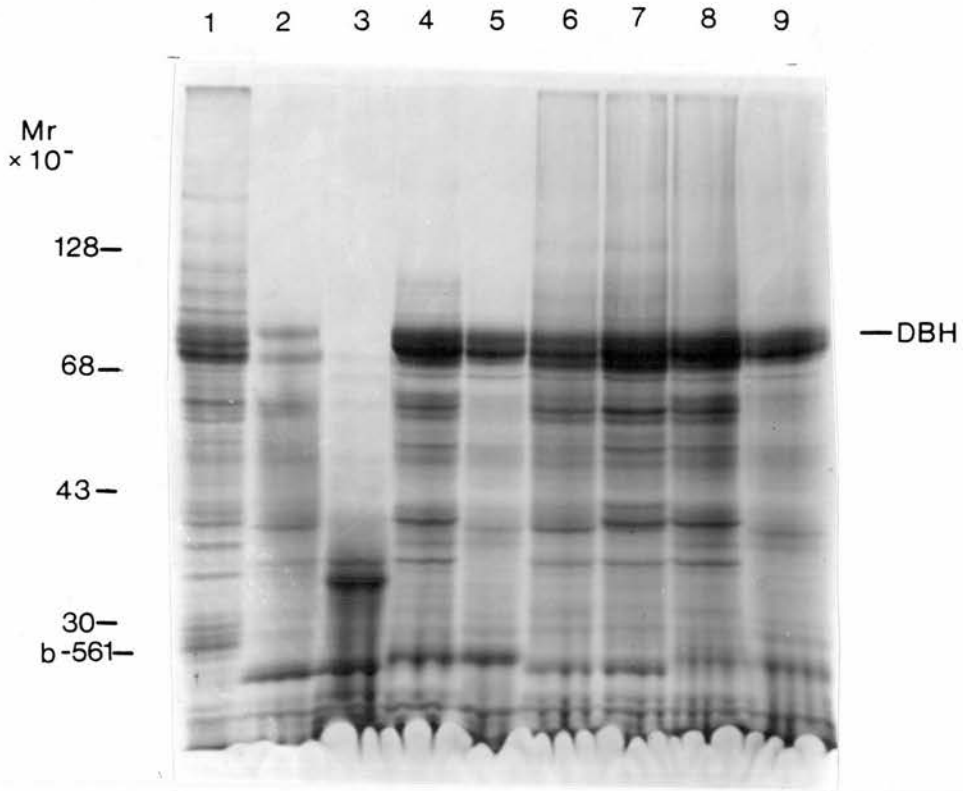


Figure 6.1 One-dimensional gel (6-15%), stained with Coomassie Blue, of membrane proteins from chromaffin granules treated with trypsin, chymotrypsin, papain or pronase. Track 1, untreated membranes (60 μ g). Tracks 2-5, membranes from protease-treated granules, prepared for electrophoresis by dissolving them in sample buffer in the presence of protease inhibitors. Tracks 6-9, membranes from protease-treated granules, prepared for electrophoresis by heating at pH 4.0 with inhibitors (see section 2.3.4). Membranes (60 μ g of protein/track) from granules treated with pronase (tracks 2 and 6), papain (tracks 3 and 7), chymotrypsin (tracks 4 and 8), trypsin (tracks 5 and 9). Abbreviations: DBH, dopamine β -hydroxylase; b-561, cytochrome b-561.

Membranes were prepared from the purified granules as described in section 2.2, and membranes were finally suspended in 10 mM Hepes pH 7.0, containing the inhibitors.

Membranes from protease-treated granules were prepared for one-dimensional electrophoresis in two ways. (1) Benzamidine (final concentration 10 mM), iodoacetamide (final concentration 10 mM) and PMSF (final concentration 2 mM) were added to the membranes in order to inactivate the proteases. Then, membranes were dissolved in sample buffer containing potassium biphthalate, pH 4.0 (section 2.3.4), to inhibit protease activity. Finally, mercaptoethanol was added (fig. 6.1, tracks 2-5). Alternatively (2), inhibitors were added, membranes were dissolved in sample buffer, pH 4.0 and then they were heated to 95°C, in order to inactivate the proteases (described in section 2.3.4). After cooling to room temperature 2-mercaptoethanol was added (fig. 6.1, tracks 6-9). It was found that heating in the presence of 2-mercaptoethanol caused extensive aggregation of membrane proteins, particularly cytochrome b-561.

It can be seen from fig. 6.1 that proteolysis by pronase and papain was not inhibited simply by adding the inhibitors and dissolving the membranes in sample buffer at pH 4.0 (tracks 2 and 3, fig. 6.1), but heating to 95°C appeared to inhibit these proteases more effectively (tracks 6 and 7, fig. 6.1). Trypsin

and chymotrypsin appeared to be reasonably inactivated merely by dissolving samples in sample buffer containing inhibitors (compare fig. 6.1, tracks 4 and 8, 5 and 9).

Cytochrome b-561 was digested by all four proteases (tracks 6-9, fig. 6.1), indicating that proteolysis had occurred on the cytoplasmic side of the granule membrane. Many bands were visible in track 1 of fig. 6.1 which could not be seen in tracks 6-9, and more bands were visible in tracks 5-7 of fig. 6.3 (membranes from intact granules treated with trypsin) than in track 9 of fig. 6.1. So, proteolysis may have occurred on the matrix side of the granule membrane as well as on the cytoplasmic side in the experiment illustrated in fig. 6.1.

In another experiment crude chromaffin granules (14 mg of protein/ml) were incubated with trypsin or chymotrypsin (100 μ g/ml) for 15 h at room temperature with gentle agitation. For the controls, three aliquots of granules were incubated in the absence of proteases for 15 h. Then, TLCK and PMSF (final concentrations 100 μ M) were added to the granules treated with trypsin; TPCK and PMSF (final concentrations 100 μ M) were added to those treated with chymotrypsin. All three inhibitors at the above concentrations were added to the controls and then trypsin (100 μ g/ml) was added to one aliquot of control granules and chymotrypsin (100 μ g/ml) to a second aliquot, to test the effectiveness of the inhibitors. Proteases were not added to the

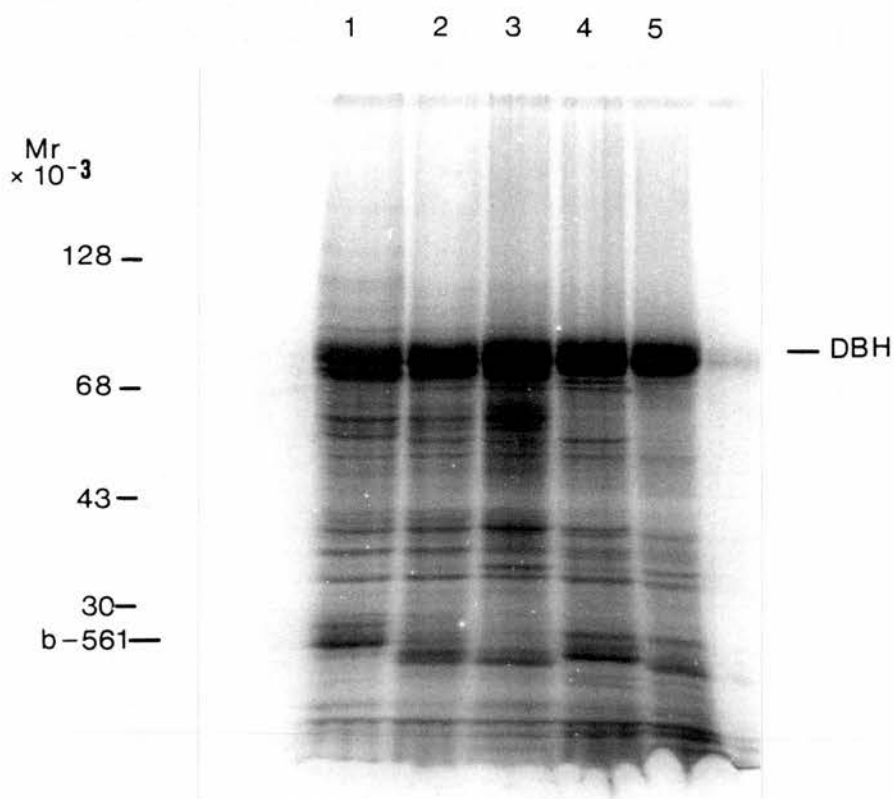


Figure 6.2 One-dimensional separation (6-15%) of membrane proteins from granules incubated with trypsin or chymotrypsin. Track 1, membranes (100 μ g) from control granules incubated for 15 h at room temperature in the absence of proteases. Track 2, membranes (100 μ g) from granules treated with chymotrypsin in the presence of 100 μ M TLCK, TPCK and PMSF. Track 3, membranes (100 μ g) from granules incubated with chymotrypsin for 15 h. Track 4, membranes (100 μ g) from granules treated with trypsin in the presence of 100 μ M TLCK, TPCK and PMSF. Track 5, membranes (100 μ g) from granules incubated with trypsin for 15 h. Abbreviations are as in fig. 6.1

third aliquot of control granules. The granules were washed by centrifugation in buffered sucrose containing inhibitors and then they were centrifuged through 1.6 M sucrose (section 2.2) to remove granules which had lysed during incubation with proteases. Membranes were prepared from the protease-treated granules as described in section 2.2, all buffers containing 100 μ M TLCK, TPCK and PMSF. Membranes were finally suspended in 10 mM Hepes containing inhibitors at the above concentrations.

Figure 6.2 shows a gel, stained with Coomassie Blue, of membrane proteins from protease-treated granules and the controls. The membranes from granules incubated for 15 h in the absence of chymotrypsin or trypsin (track 1, fig. 6.2) were almost identical with untreated membranes (track 1, fig. 6.1), indicating that proteins were not digested by endogenous proteases found in granules. However, some degradation of proteins occurred in granules treated with chymotrypsin or trypsin in the presence of inhibitors (fig. 6.2: compare tracks 2 and 4 with track 1). In these experiments cytochrome b-561 is a particularly sensitive indicator of proteolysis, although proteolysis may have occurred on the matrix side of the membrane as well as the cytoplasmic side after lysis of the granules (section 2.2). Not surprisingly proteolysis was more extensive in the granules incubated for 15 h with proteases (tracks 3 and 5, fig. 6.2). Therefore in

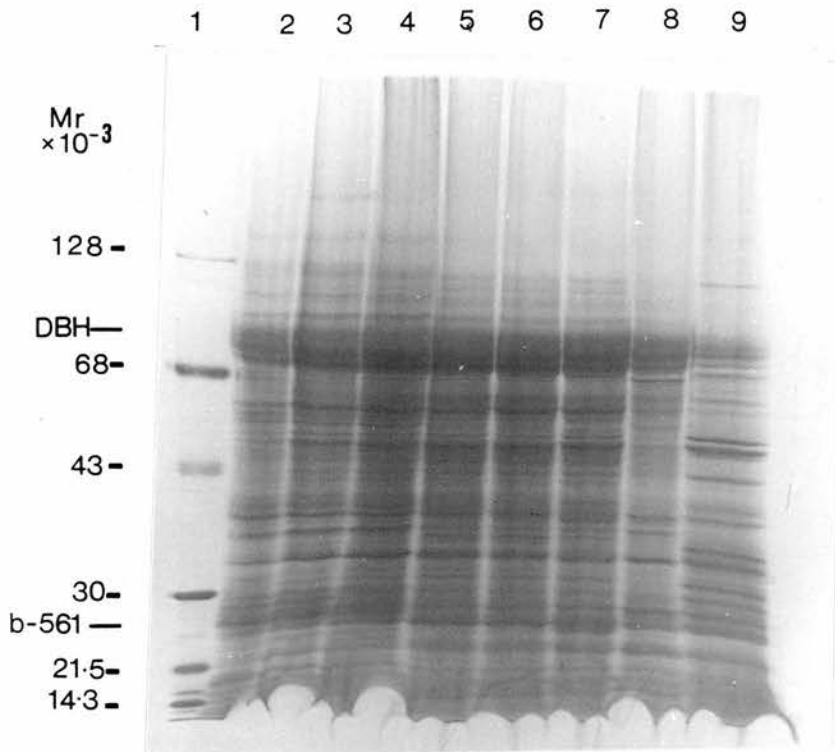


Figure 6.3 Electrophoretic analysis of membrane proteins from granules treated with trypsin. The gel (6-15%) was stained with Coomassie Blue. 100 μ g of membrane protein was loaded per track. Track 1, standard proteins were as in section 2.15.4. Track 2, untreated chromaffin granule membranes. Track 3, membranes from granules incubated in the absence of trypsin for 3 h. Track 4, membranes from granules incubated with trypsin in the presence of trypsin inhibitor. Tracks 5-7, membranes from granules incubated with trypsin for 30 min (track 5), 1 h (track 6) and 3 h (track 7). Track 8, broken membranes treated with trypsin. Track 9, membranes from granules treated with Triton X-100 and then incubated with trypsin.

this experiment chymotrypsin and trypsin were not completely inactivated by the treatment with TPCK, TLCK and PMSF.

The method eventually adopted for treating intact granules with trypsin (50 $\mu\text{g/ml}$) is described in section 2.2.2; incubations were for 30 min, 1 h or 3 h at room temperature and then trypsin inhibitor (100 $\mu\text{g/ml}$) was added to inactivate the enzyme. For the controls, granules were incubated for 3 h in the absence of trypsin followed by the addition of trypsin inhibitor (100 $\mu\text{g/ml}$) or trypsin (50 $\mu\text{g/ml}$) which had been mixed with trypsin inhibitor (100 $\mu\text{g/ml}$). Intact granules were then purified by centrifugation through 1.6 M sucrose as before, and membranes were prepared from them. Experiments with broken membranes were done by subjecting a preparation of granule membranes to two cycles of freezing (at -20°C) and thawing (at room temperature) in the presence of trypsin (50 $\mu\text{g/ml}$) over 90 min. In addition, granules were treated with 1% w/v Triton X-100 and then incubated with trypsin. Membranes were prepared from these damaged granules by centrifugation at 45000 r.p.m. (section 2.2.2), and soluble proteins were found in the supernatant from this step, which was discarded. Proteins were prepared for SDS polyacrylamide gel electrophoresis as described in section 2.3.4.

Figure 6.3 clearly shows that no proteolysis occurred in granules incubated for 3 h at room

temperature or when granules were treated with trypsin in the presence of trypsin inhibitor (compare tracks 2 and 3 with track 1). In particular, cytochrome b-561 which is highly susceptible to proteolysis (Hunter et al., 1982) was not digested, suggesting that any proteolysis that occurred in the trypsin-treated granules had to be a result of digestion of proteins on the exterior (cytoplasmic) surface of the granule membrane, and could not be arising artefactually during the period of sample preparation after the digestion period. Inspection of fig. 6.4 shows that cytochrome b-561 was cleaved to form a polypeptide migrating with an apparent Mr of 25000 in tracks 4, 5 and 6. Many bands were still visible in the gel after treating broken membranes with trypsin (track 8) suggesting either that membrane vesicles had formed (in spite of freezing and thawing) which restricted the accessibility of trypsin to proteins, or that many polypeptides are protected from extensive proteolysis when folded in their native conformations in the membrane. When granules solubilized with 1% w/v Triton X-100 were incubated with trypsin many polypeptides were degraded, the most notable being DBH (track 9, fig. 6.3). A broad band of DBH (Mr 75000) was still visible after the incubations of intact granules with trypsin (tracks 5, 6, 7, fig. 6.3) suggesting that DBH may be exposed only on the matrix surface of the membrane. Several prominent polypeptides appeared to be resistant

Figure 6.4 Autoradiographs of replicas decorated with (A) anti-membrane DBH serum (diluted 100-fold); (B) anti-cytochrome b-561 serum (diluted 200-fold); (C) anti-glycoproteins J and K serum (diluted 100-fold); (D) anti-glycoprotein lll serum (diluted 100-fold). Track 1, untreated chromaffin granule membranes (100 μ g). Track 2, membranes from granules incubated for 3 h at room temperature in the absence of trypsin. Track 3, membranes from granules treated with trypsin in the presence of trypsin inhibitor. Tracks 4-6, membranes from granules incubated with trypsin for 30 min (track 4), 1 h (track 5) and 3 h (track 6). Track 7, broken membranes incubated with trypsin. Track 8, membranes from granules treated with 1% w/v Triton X-100 and then incubated with trypsin (not shown in B).

The mark indicated by the arrow in (B) and the absence of bands in track 1 of (D) resulted from uneven contact of the cellulose nitrate sheets with the gels during transfer of proteins. The two bands indicated by arrows in (C) are probably proteolysis products of glycoproteins J and K. The identity of bands X (Mr 75000) and Y (Mr 64000-66000) is discussed in section 4.6.

Abbreviations: b-561, cytochrome b-561; DBH, dopamine β -hydroxylase; Gplll, glycoprotein lll.

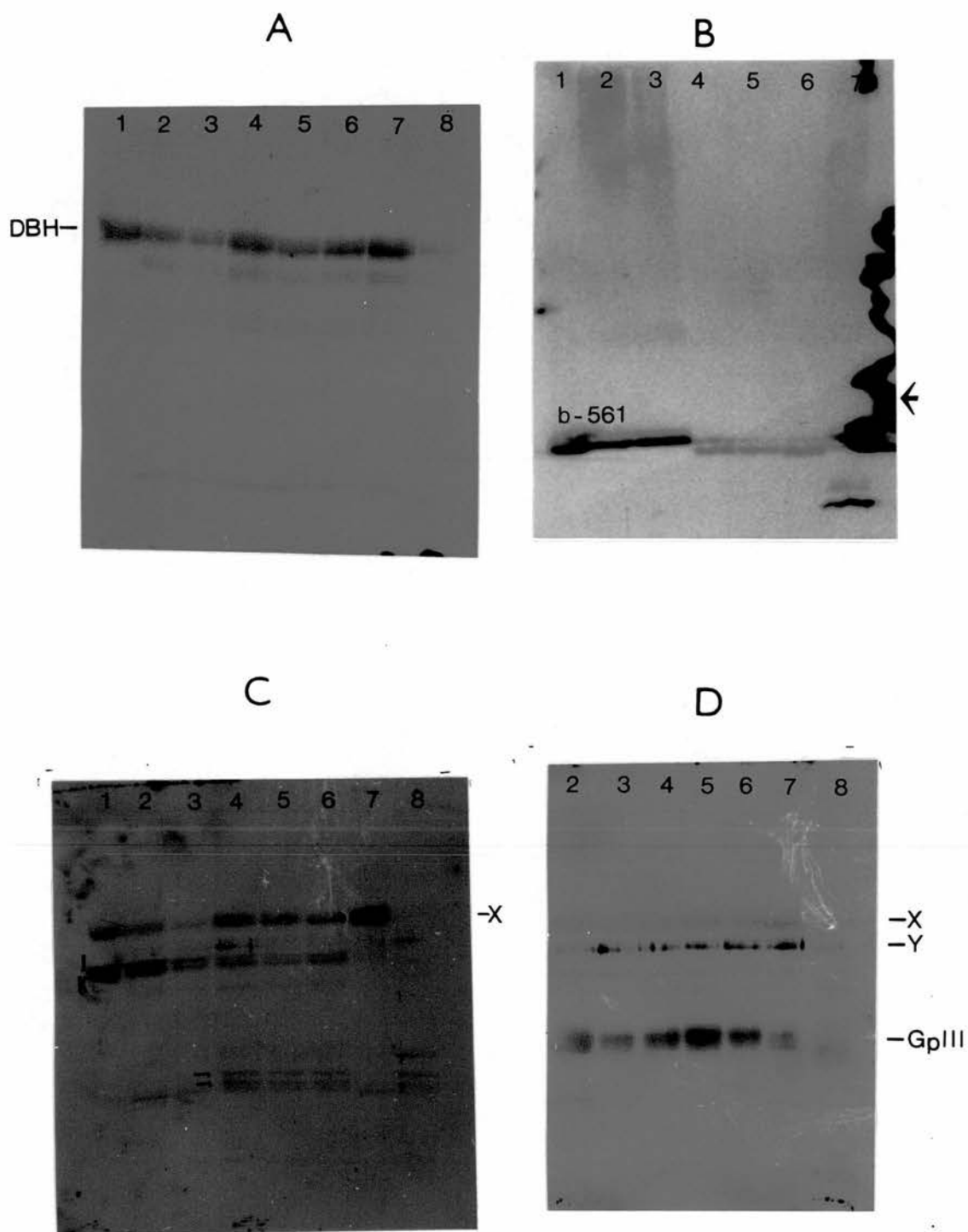


Figure 6.4

to trypsin in the presence of Triton X-100, and several new polypeptides were generated. These could be proteolysis products of membrane glycoproteins which might appear as sharply focussed bands once all the carbohydrate had been removed. An unequivocal way of identifying these bands would be to decorate replicas of gels with antisera against the deglycosylated glycoproteins but such antisera were not available.

Cellulose nitrate replicas of gels prepared as in fig. 6.3 were decorated with antisera against DBH, cytochrome b-561, Gplll and glycoproteins J and K. Autoradiographs of these replicas are presented in figure 6.4. Cytochrome b-561, DBH, Gplll and glycoproteins J and K were not digested in the controls (compare tracks 2 and 3 with track 1 in each case). Because samples containing proteases could not be loaded onto the gel using a Hamilton syringe, slightly uneven loading of the gel resulted in the bands in tracks 2 and 3 of fig. 6.4A being fainter than in tracks 1, 4 and 5.

In the case of DBH a broad band (apparent Mr 65000) was visible after digestion of intact granules and broken membranes with trypsin and in the controls (tracks 2-7, fig.6.4A), suggesting that some intra-granular proteolysis had occurred. However, the band apparent Mr 75000 only decreased in intensity in the presence of Triton X-100 (track 9, fig. 6.3; track 8, fig. 6.4 A), and it was noteworthy that no tryptic

fragments binding antibody were visible in track 8 (fig. 6.4A) in the Mr range 20000 - 75000.

The replica decorated with anti-cytochrome b-561 serum is shown in fig. 6.4B. The major band of apparent Mr 27000 has a faint band above it, which has often been seen previously, and the significance of which is unknown. On trypsin digestion from outside the granules these bands become fainter to be replaced by a band of apparent Mr 25000, which still binds antibody, unlike the pronase digestion product of Hunter et al. (1982). This product is not formed to any great extent in broken membranes; rather, the transmembrane protein is degraded to a species of apparent Mr 18000 which also has a minor band just above it. In the presence of Triton X-100 (not shown) a faint band of the mature protein (apparent Mr 27000) and the digestion product (apparent Mr 25000) were visible; although the band Mr 25000 was much fainter than in tracks 4-6 of fig. 6.4B.

Gplll was not visible in track 1 of fig. 6.4D because of uneven contact of the cellulose nitrate sheet with the gel during transfer of proteins, but the glycoprotein was not digested in the controls (tracks 2, 3). Gplll was not digested when intact granules were treated with trypsin (tracks 4-6, fig. 6.4D). However, when broken membranes were treated there ^{may have been} a slight decrease in the apparent Mr of Gplll (track 7) and solubilization with Triton X-100 resulted in the appearance of a new band of apparent

Mr 29000-34000 (track 8).

Anti-glycoproteins J and K serum labelled two new bands of apparent Mr 27000 and 30000 (approximate values) after digesting intact granules with trypsin (tracks 4-6, fig. 6.4C). The antiserum additionally decorated a band of apparent Mr 26000 in the controls (tracks 2 and 3), and in broken membranes (track 7), suggesting that there may have been some intragranular proteolysis in this case. In broken membranes the GpJ and GpK bands (apparent Mr 57000 and 53000) and ^{possible} the digestion products at 27000 and 30000 were susceptible to trypsin, suggesting that there are cleavage sites on both sides of the membrane. Some of the 27000 and 30000 bands were visible after treatment in the presence of Triton X-100. So, these results indicate that glycoproteins J and K have trypsin-sensitive sites on both sides of the membrane and so they could be transmembrane glycoproteins. As pointed out by Huber et al. (1979) and Eagles et al. (1975), Con A does not bind to intact granules and so the oligosaccharides of glycoproteins J and K are likely to be on the inner surface of the granule membrane.

6.3 Location of glycoprotein lll, glycoprotein J and glycoprotein K in bovine brain

The pituitary gland, cortex and basal ganglia

Figure 6.5 One-dimensional gels (6-15%) of proteins from bovine brain. (A) Stained with Coomassie Blue. (B) Autoradiograph of a replica decorated with anti-glycoproteins J and K serum (diluted 50-fold). (C) Autoradiograph of a replica decorated with anti-glycoprotein lll serum (diluted 50-fold). Track 1, chromaffin granule membranes (80 μ g). Track 2, pituitary (100 μ g in A; 200 μ g in B and C). Track 3, basal ganglia (100 μ g in A; 200 μ g in B and C). Track 4, cortex (100 μ g in A; 200 μ g in B and C). Bands x-z in (A) probably correspond with the same bands in (B). The identity of band e in track 1 of (B) is not known. Abbreviations; DBH, dopamine β -hydroxylase; b-561, cytochrome b-561; Gplll, glycoprotein lll.

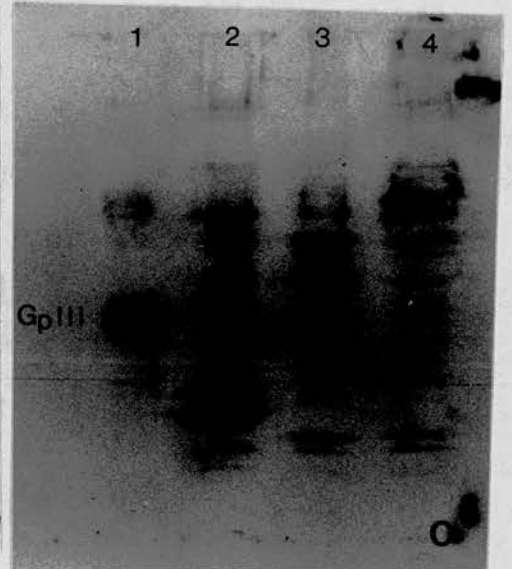
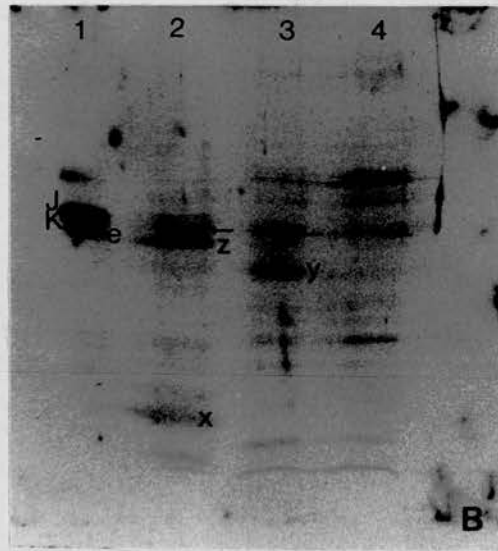
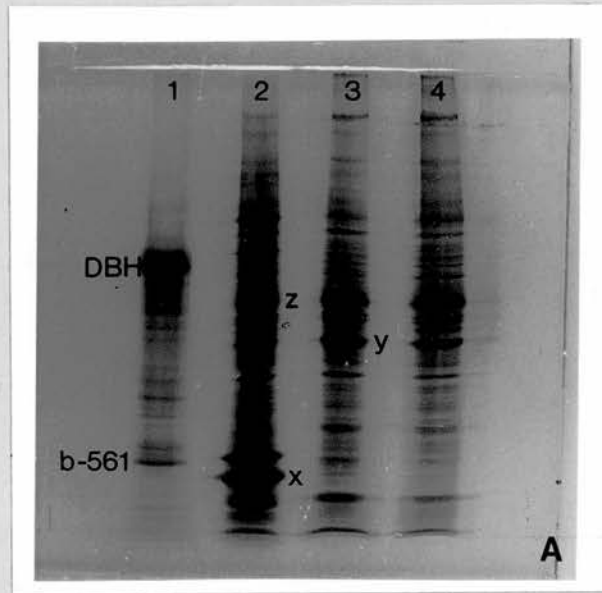


Figure 6.5

from bovine brain were homogenized in 10 mM Hepes, pH 7.0 and then membranes were prepared from these tissues by centrifugation at 4000 r.p.m. (1540 g) for 5 min followed by centrifugation of the supernatant from this step at 45000 r.p.m. (158000 g) for 30 min (see section 2.2 for rotors). The pellets of membranes were resuspended in 10 mM Hepes, pH 7.0 and proteins were analysed by one-dimensional electrophoresis under reducing conditions. Figure 6.5A shows that the pituitary contains proteins that are not found in the basal ganglia, cortex or in chromaffin granule membranes. The pattern of bands in tracks 3 and 4 of fig. 6.5A are very similar indicating that some polypeptides are common to both the cortex and basal ganglia.

To investigate if glycoproteins J and K are found in the brain, proteins were transferred to cellulose nitrate and the replica was decorated with anti-glycoproteins J and K serum. Gplll is found in the pituitary (Fischer-Colbrie et al., 1984) so for the control a replica was decorated with anti-Gplll serum. Autoradiographs of the replicas are illustrated in figure 6.5 (B and C). The gels were heavily loaded with protein (200 µg/track) and the antisera were only diluted 50-fold, instead of 100-fold as in previous experiments. This resulted in non-specific binding of antisera to proteins and consequently many bands were visible on the autoradiographs. Nevertheless, a band

which may correspond to Gp111 could be seen in the track containing proteins from the pituitary (compare track 2 with track 1, fig. 6.5C), though not in the other tracks.

A band migrating to the same region of the gel as GpK (indicated by arrows in fig. 6.5B) was visible in the tracks containing pituitary, basal ganglia and cortex. A very faint band of similar apparent Mr to GpJ could be detected in track 2 of fig. 6.5B. The identity of band e (track 1, fig. 6.5B) is not known and has not been seen previously. In summary, it is very difficult to determine whether glycoproteins J and K are found in brain from this experiment because of non-specific binding of the anti-glycoproteins J and K serum to other proteins on the replica.

6.4 Localization of glycoprotein 111, dopamine β -hydroxylase, glycoproteins J and K in isolated chromaffin cells by immunofluorescence microscopy

Isolated chromaffin cells were incubated with antisera against Gp111, glycoproteins J & K and DBH, and binding of antibodies was detected by immunofluorescence microscopy. Fluorescence micrographs and their corresponding phase pictures (figs. 6.6, 6.7, 6.8) were made by Dr. J. H. Phillips. Chromaffin cells are small, round and often attach to contaminating endothelial cells (fig. 6.6A). They have dense nuclei and the phase-dense granules give their cytoplasm a distinct grey appearance. Endothelial cells are larger and clearly spread well on the coverslips (figs. 6.6A, 6.7B,

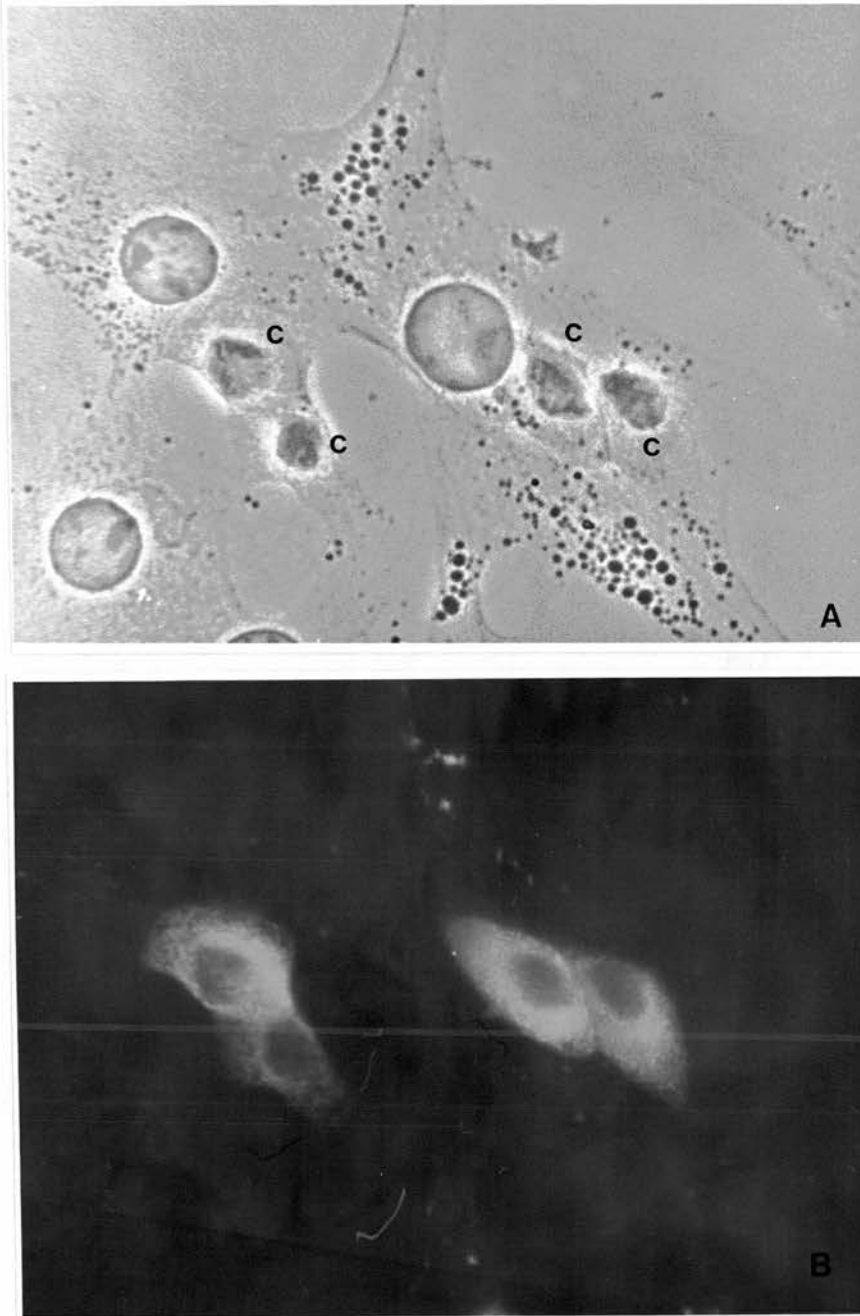


Figure 6.6 Immunofluorescent staining of chromaffin cells with anti-membrane DBH serum (diluted 25-fold). (A) Phase picture. Chromaffin cells (indicated by c) are small, round and sit on top of endothelial cells. (B) Corresponding fluorescence picture. Only the chromaffin cells show detectable fluorescence. Magnification x900.

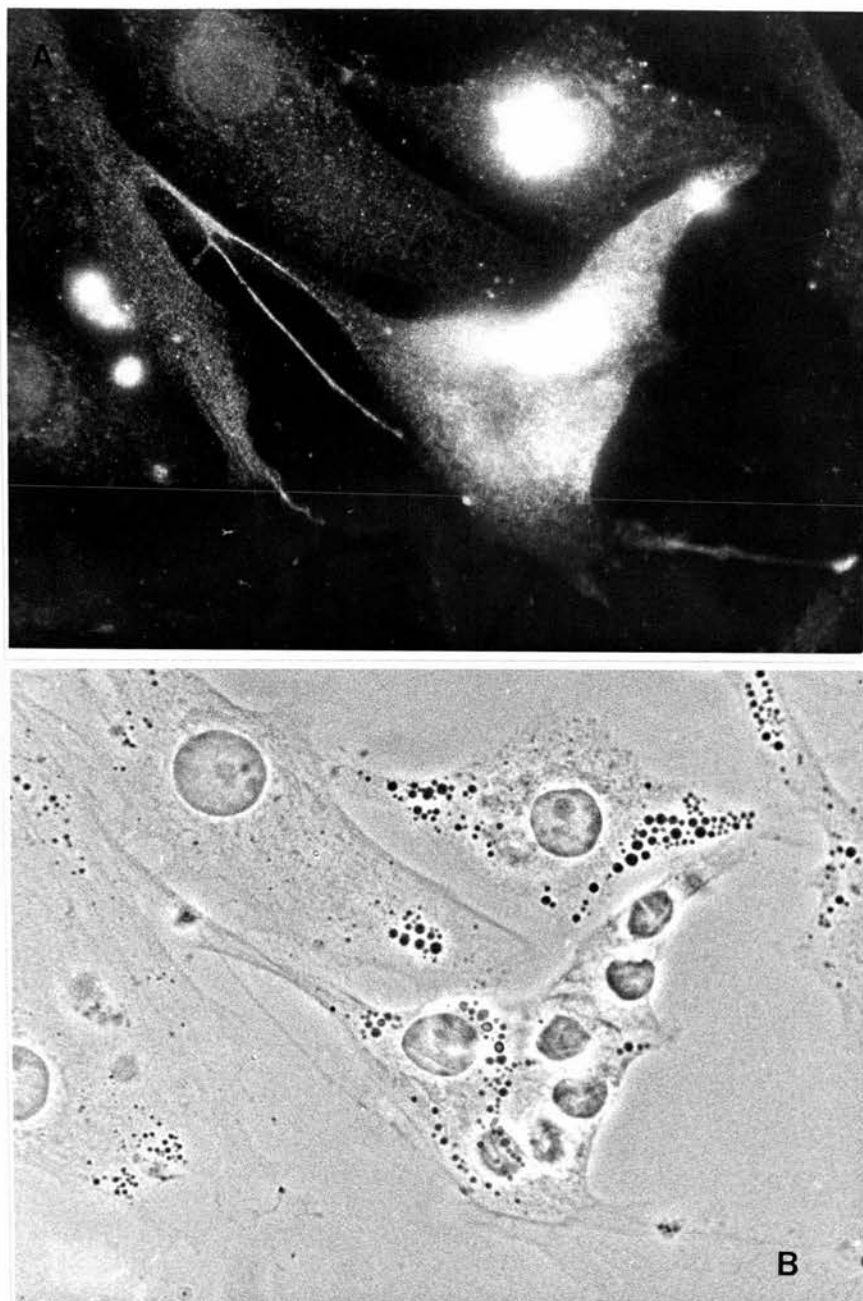


Figure 6.7 Binding of anti-glycoproteins J and K serum (diluted 25-fold) to chromaffin cells. (A) Immunofluorescence staining of chromaffin cells with the antiserum. (B) Phase picture corresponding to the fluorescence micrograph. The chromaffin cell that is fluorescent at the top of the picture in (A) is out of phase in (B) because it lies on top of an endothelial cell. Magnification x900.

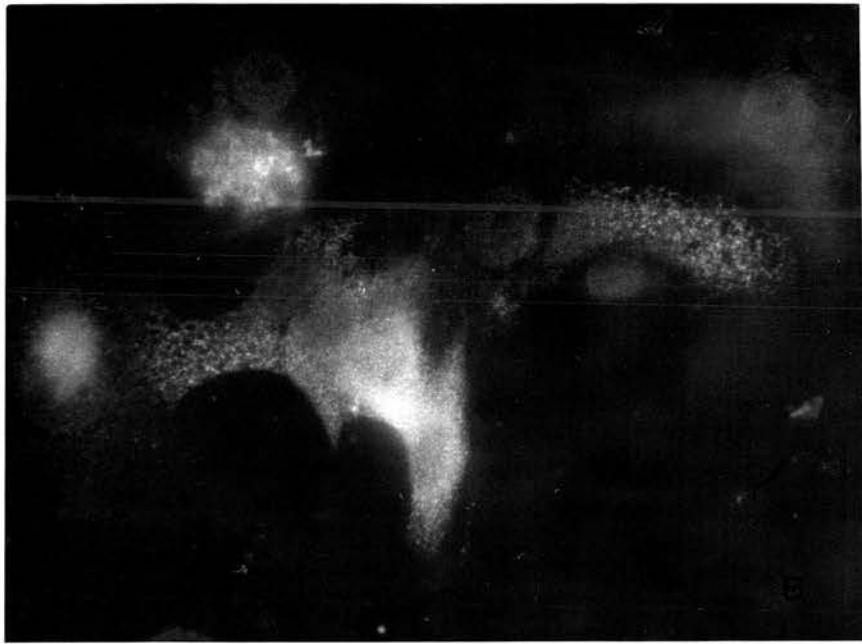
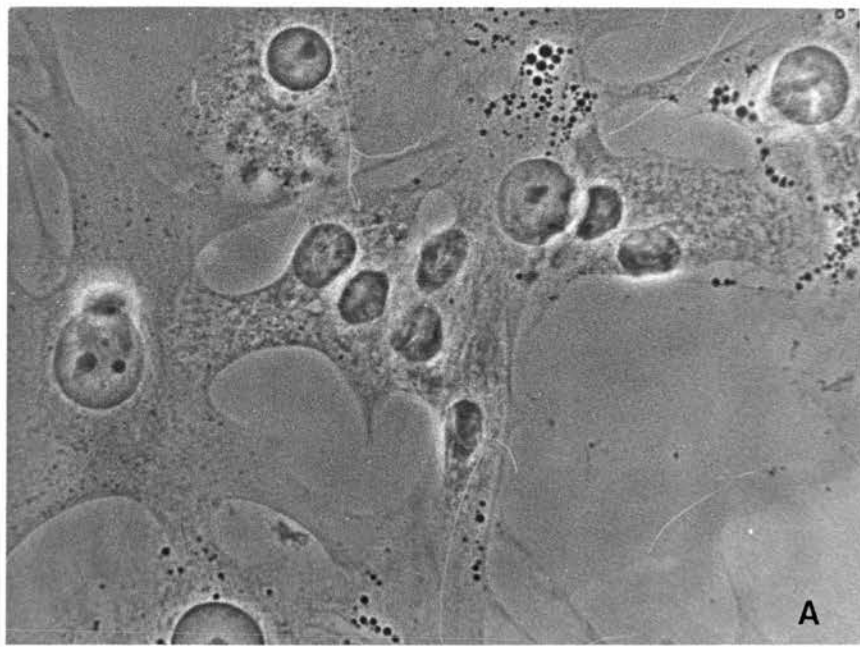


Figure 6.8 Immunofluorescence of chromaffin cells with anti-glycoprotein 111 serum (diluted 25-fold). (A) Phase picture. (B) Corresponding fluorescence picture. The fluorescent staining of chromaffin cells, which are more spread out, is very granular in appearance. Magnification x900.

6.8A). All antisera labelled chromaffin cells but not endothelial cells, although the reaction of anti-membrane DBH serum with chromaffin cells was much weaker (compare fig. 6.6B with 6.7A and 6.8B). The fluorescence was confined to the cytoplasm and where the cells are spread out (fig. 6.8B) fluorescence was granular in appearance, suggesting that the antigens are found in chromaffin granules.

CHAPTER SEVEN
MONOCLONAL ANTIBODIES

7.1 Introduction

Secretion of proteins is highly regulated in exocrine, endocrine and neuronal cells. The secretory proteins are stored in vesicles and are released by exocytosis (reviewed by Orci and Perrelet, 1978; Meldolesi et al., 1978). Examples of such secretory vesicles are : β -secretory granules from endocrine cells of pancreas (Orci and Perrelet, 1978; Orci et al., 1984); secretory vesicles of parotid and lacrimal glands (Castle et al., 1975; Herzog et al., 1976); synaptic vesicles of the nervous system and of neuromuscular junctions (Heuser and Reese, 1981; Kelly et al., 1979, 1983); chromaffin granules in the adrenal medulla. In most cases, the major secretory components of these vesicles have been well characterized, but information on the membrane proteins is limited. Membrane proteins are hard to purify, they are often present in small amounts, some have no easily measured biological activity or else their activity is destroyed when the membranes are solubilized for analysis. Nevertheless, monoclonal antibodies to membrane proteins can be used to locate membrane proteins in subcellular fractions and could therefore provide a means for studying granule biogenesis and the biochemical mechanism of exocytosis.

Monoclonal antibodies were first produced by Köhler and Milstein (1975) and they have many industrial and medical applications. Several proteins from the membrane

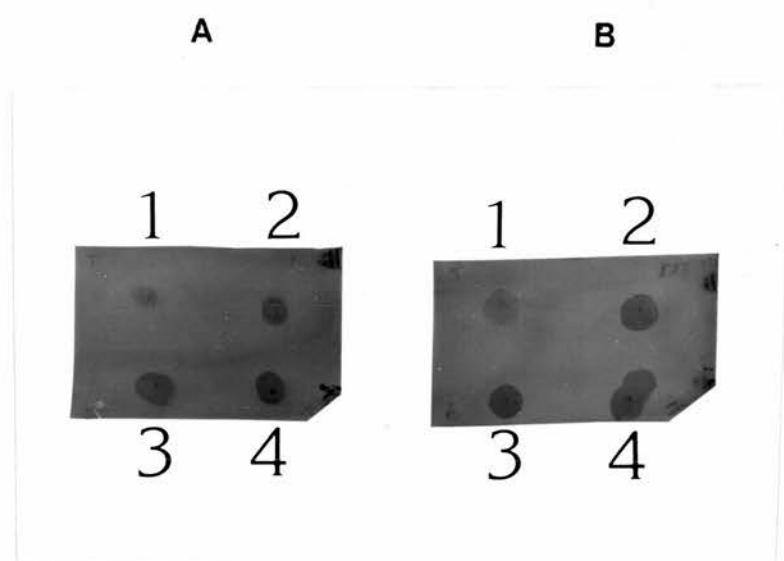


Figure 7.1 Immunodotting assay for monoclonal antibodies. Rectangles of cellulose nitrate were incubated with lysate (A) or chromaffin granule membranes (B) and then supernatants from a culture of hybridoma cells were dotted onto the rectangles. 1-4 indicate supernatants taken from the cell culture at different times; 4 being taken from the cells after a longer growth period. Binding of monoclonal antibodies was detected by incubation with HRP-conjugated sheep anti-mouse IgG, followed by staining with chloronaphthol stain (section 2.13.1).

of synaptic vesicles have been found in the adrenal medulla using monoclonal antibodies. These proteins include p65 (Mr 65000; Matthew et al., 1981); synaptophysin (Mr 38000; Wiedenmann and Franke, 1985) and SV2 (Buckley and Kelly, 1985).

Monoclonal antibody A4, which was shown to recognize DBH, was produced in collaboration with Dr. J. Haywood (method described in section 2.12). The clones were screened by immunohistochemical methods (carried out by Dr. Haywood) and by the immunodotting assay (section 2.13.1). A method for detecting the binding of monoclonal antibodies to cellulose nitrate replicas of chromaffin granule membrane proteins was developed (section 2.13.2).

7.2 Characterization of monoclonal antibody A4

Supernatants from cultures of hybridoma cells were tested for the secretion of antibodies by the immunodotting assay (described in section 2.13.1). Rectangles of cellulose nitrate were incubated with chromaffin granule membranes or lysate (2 mg of protein/ml), then dots of supernatants taken from a cell culture at different times were placed on the rectangles. After washing, binding of monoclonal antibodies was detected by incubation with HRP-conjugated sheep anti-mouse IgG (SAM-HRP) followed by staining with a solution containing chloronaphthol and hydrogen peroxide (section 2.13.1).

Figure 7.1 shows that the dots of culture supernatants were stained dark grey indicating the presence of a monoclonal antibody (A4) to a membrane

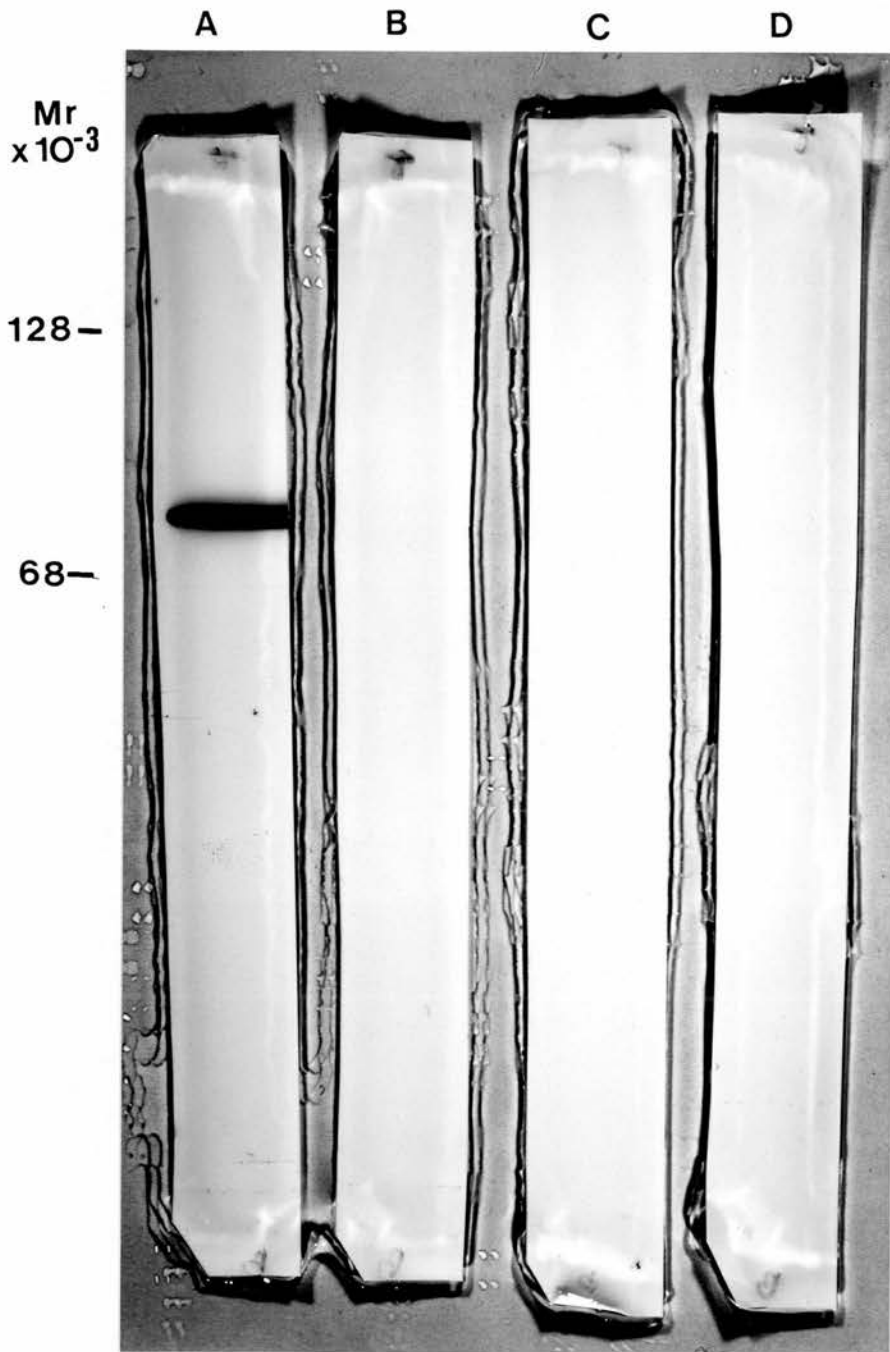


Figure 7.2 Cellulose nitrate replicas of: (A and B) chromaffin granule membrane proteins - 80 μ g per track; (C and D) lysate proteins - 80 μ g per track. The replicas were incubated with : (B and D) serum buffer - see section 2.13.2 or with (A and C) culture supernatant containing antibody A4, diluted 5 - fold in serum buffer.

and matrix component. Not surprisingly, the supernatant taken from the cells at an early stage (dot 1 in fig. 7.1) did not react as well as supernatant (dot 4 in fig. 7.1) taken from the cells after a longer growth period.

To identify the antigen recognized by antibody A4 the immune replica technique was used with some modifications (described in section 2.13.2). Denaturation of proteins by SDS interferes with the binding of some monoclonal antibodies (Riezman, 1984) so proteins were transferred from SDS gels to cellulose nitrate sheets for 6 h instead of for 90 min and SDS was omitted from the transfer buffer (0.025 M Na_2HPO_4 , 20% v/v methanol). The replicas were blocked by incubation with Tween 20 buffer (section 2.13) instead of BSA buffer (section 2.5). The background on the replicas was much lower when the blocking buffer contained Tween 20. Culture supernatant or ascites fluids (see later) containing monoclonal antibodies were diluted in a buffer containing 5% v/v normal sheep serum or heat inactivated horse serum (serum buffer in section 2.13.2).

Figure 7.2 shows cellulose nitrate replicas of membrane and lysate proteins (reduced with 2-mercaptoethanol) decorated with culture supernatant (diluted 5-fold) containing antibody A4. Antibodies in normal sheep serum did not bind to membrane or lysate proteins (B and D, Fig. 7.2) neither did antibodies in heat-inactivated horse serum (results not shown). Monoclonal antibody A4 recognized a membrane protein

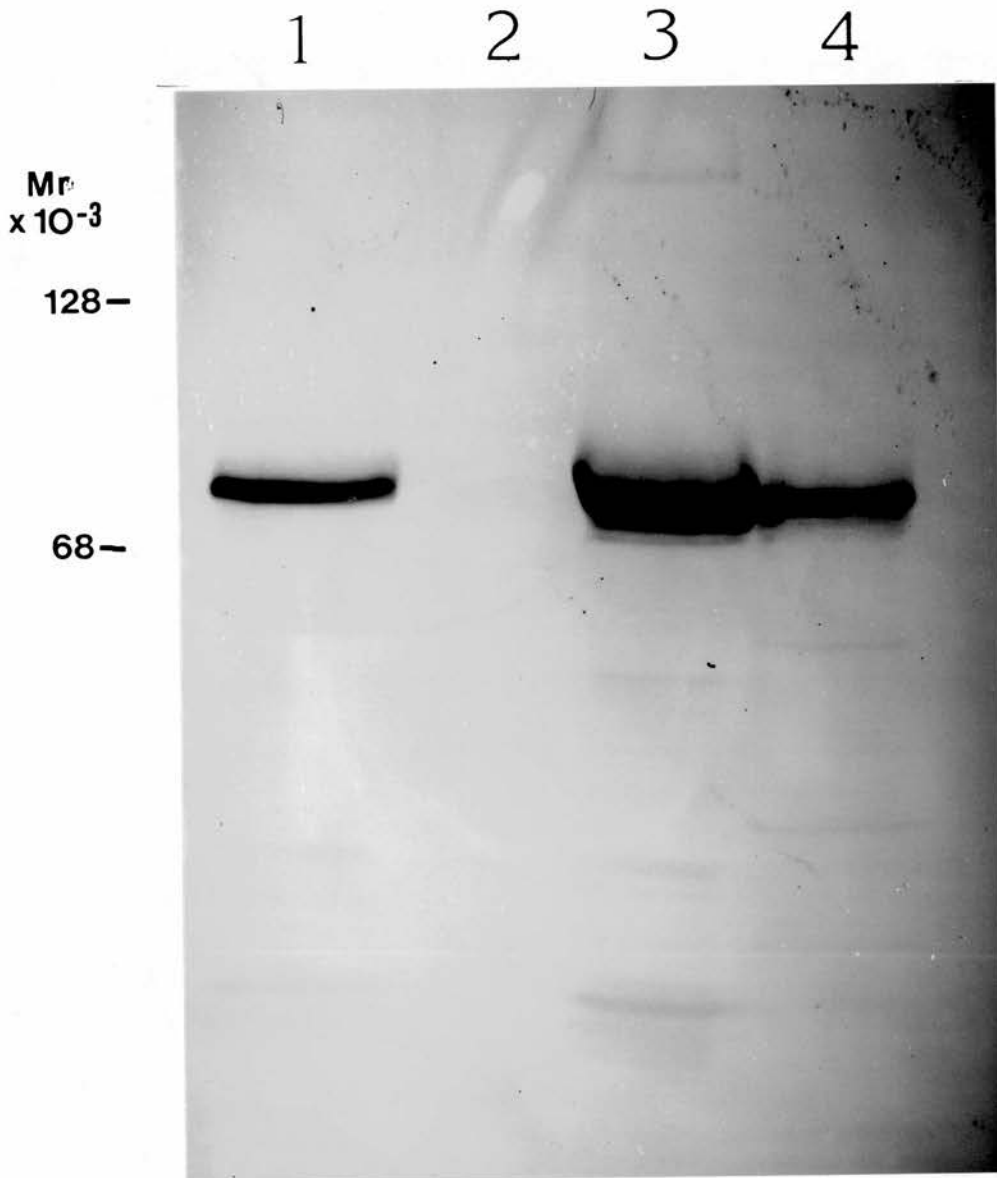


Figure 7.3 Cellulose nitrate replica decorated with culture supernatant containing antibody A4 (diluted 5-fold). Track 1, chromaffin granule membranes (150 μ g). Track 2, lysate (150 μ g). Track 3, detergent-rich phase obtained after solubilization and temperature induced phase separation of chromaffin granule membrane proteins in Triton X-114 (150 μ g, gift of J. G. Pryde). Track 4, eluate from Con A - agarose.

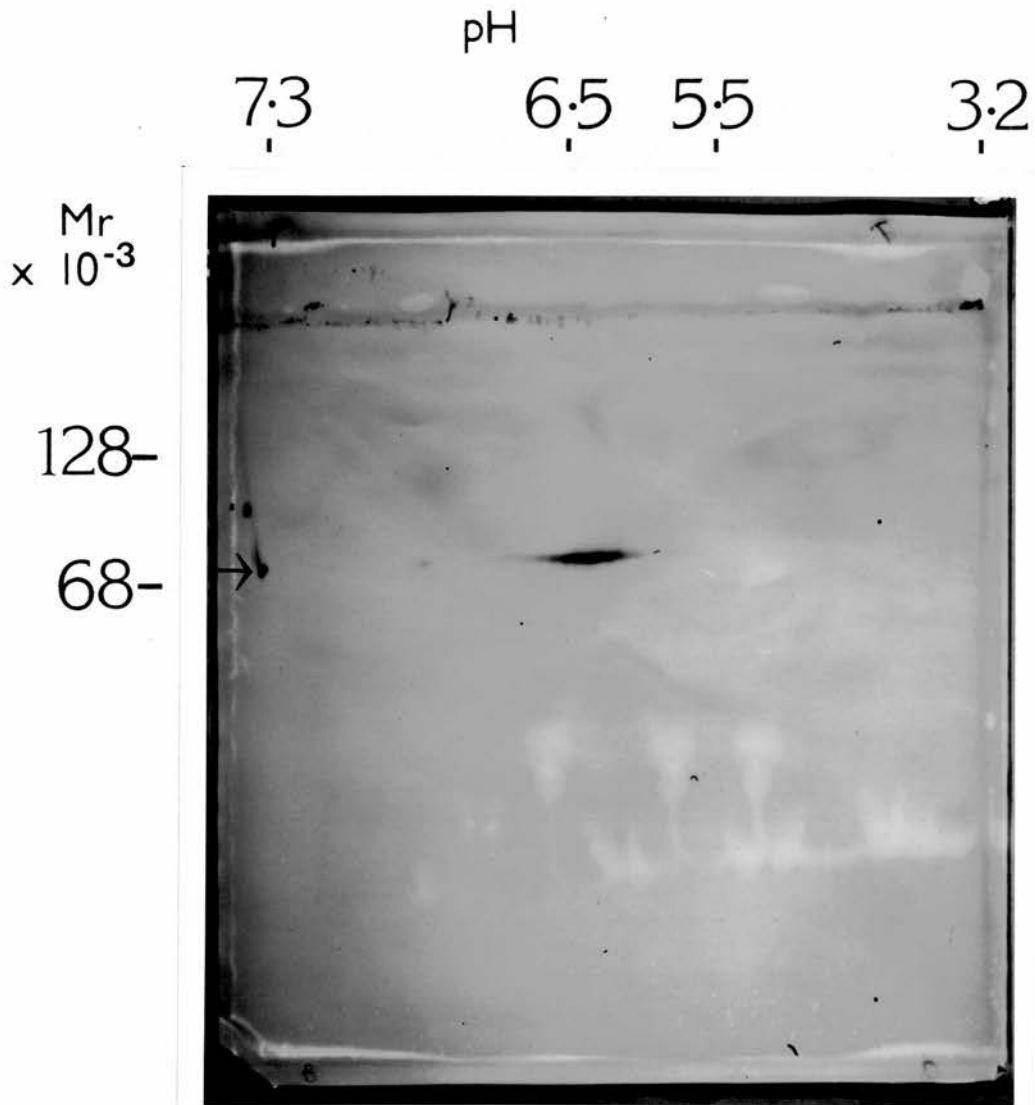


Figure 7.4 Cellulose nitrate replica of a two-dimensional gel of chromaffin granule membranes (300 μ g) decorated with culture supernatant containing antibody A4. \rightarrow indicates DBH which has precipitated at the top of the isoelectrofocussing gel.

of apparent Mr 74000 (A, fig. 7.2) but it did not appear to react with any protein in the soluble lysate (C, fig. 7.2).

To further characterize the antigen recognized by antibody A4 a cellulose nitrate replica of several fractions was decorated with culture supernatant (fig. 7.3). Chromaffin granule membrane proteins were fractionated into three phases by temperature-induced phase separation in Triton X-114 (Pryde and Phillips, 1986); the detergent-rich phase contains membrane DBH. The eluate from a separation of solubilized membrane proteins on Con A - agarose (see chapter 3) also contained DBH. Figure 7.3 shows that a single protein band of apparent Mr 74000 was labelled by A4 in the tracks containing chromaffin granule membranes (track 1), the detergent-rich phase from the Triton X-114 separation (track 3) and the eluate from Con A - agarose (track 4). Because all these fractions contain DBH, monoclonal antibody A4 apparently recognized this glycoprotein. However, antibody A4 did not appear to react with reduced lysate proteins, (track 2, fig. 7.3) in contrast to its reaction with a lysate component when tested by the immunodotting assay (fig. 7.1).

A replica of a two-dimensional gel of chromaffin granule membranes decorated with culture supernatant containing antibody A4 is illustrated in fig. 7.4. The antibody recognized the middle of the three DBH components recognized by polyclonal sera (Mr 74000, pI 6.0-6.8), called component F by Gavine et al. 1984).

This appeared as a series of closely-joined spots, forming a streak over a quite wide pH range.

Soluble DBH purified by affinity chromatography (gift of Dr. D. K. Apps), purified membrane DBH (chapter 3), and soluble DBH deglycosylated by trifluoromethanesulphonic acid (gift of Dr. D. K. Apps) were electrophoresed in a one-dimensional gel. The proteins were transferred to a cellulose nitrate sheet which was then decorated with culture supernatant containing antibody A4. Figure 7.5 shows that A4 recognized purified soluble DBH (track 3), purified membrane DBH (track 5) as well as DBH in chromaffin granule membranes (track 1). The gel was overloaded with purified soluble DBH, and this produced three labelled bands (track 3). Deglycosylated soluble DBH migrates with an apparent Mr of 72000 (Apps et al., 1985) but A4 reacts with a component of Mr 74000 in track 4. After photography, the replica shown in fig. 7.5 was incubated with BSA buffer (section 2.5), then with polyclonal anti-membrane DBH serum and autoradiographed. Deglycosylated soluble DBH (Mr 72000; Apps et al., 1985) was labelled by the polyclonal antiserum (not shown), so A4 must be reacting with residual glycosylated DBH in track 4 of fig. 7.5.

Monoclonal antibody A4 labelled both soluble and membrane DBH but it did not react with DBH in the lysate reduced by 2-mercaptoethanol (figs. 7.2, 7.3 and 7.5). Chromogranin A is the major protein of the granule lysate, and is at least ten times as abundant as soluble DBH (Winkler, 1976)



Figure 7.5 Replica of a one-dimensional gel (6-15%) decorated with culture supernatant containing antibody A4. Track 1, chromaffin granule membranes (150 μ g). Track 2, lysate (150 μ g). Track 3, purified soluble DBH (60 μ g). Track 4, deglycosylated soluble DBH (40 μ g). Track 5, purified membrane DBH.

It migrates to a region of a one-dimensional gel only slightly below soluble DBH under reducing conditions, so it is possible that it is masking the epitope recognized by antibody A4 in the blots of whole lysate; alternatively 2-mercaptoethanol itself might inhibit binding. To test these possibilities, reduced and unreduced lysate proteins were electrophoresed in separate SDS gels, since in the absence of reducing agent DBH migrates as a dimer of Mr 150000. Replicas were then decorated with supernatant containing antibody A4. Figure 7.6 shows that antibody A4 reacted with unreduced soluble DBH (apparent Mr 150000; fig. 7.6A) and this protein (and no other) was labelled when 50-200 µg of soluble protein was loaded onto the gel (tracks 1-4). Antibody A4 did not react with soluble DBH in lysate reduced with 2-mercaptoethanol (fig. 7.6B) but it recognized DBH in 2-mercaptoethanol-reduced membranes (track 5, fig. 7.6B) from which chromogranin A had mostly been removed. Faint bands of DBH were, however, seen on replicas of lysate reduced with dithiothreitol, suggesting that 2-mercaptoethanol may also have inhibited binding. Chromogranin A focusses at a very different pI from DBH on two-dimensional electrophoresis and soluble DBH was labelled by antibody A4 after transfer of reduced lysate proteins from a two-dimensional gel to cellulose nitrate (not shown). Thus it seems likely that the presence of chromogranin A, in the region of the DBH band, inhibited the binding of A4 in replicas of

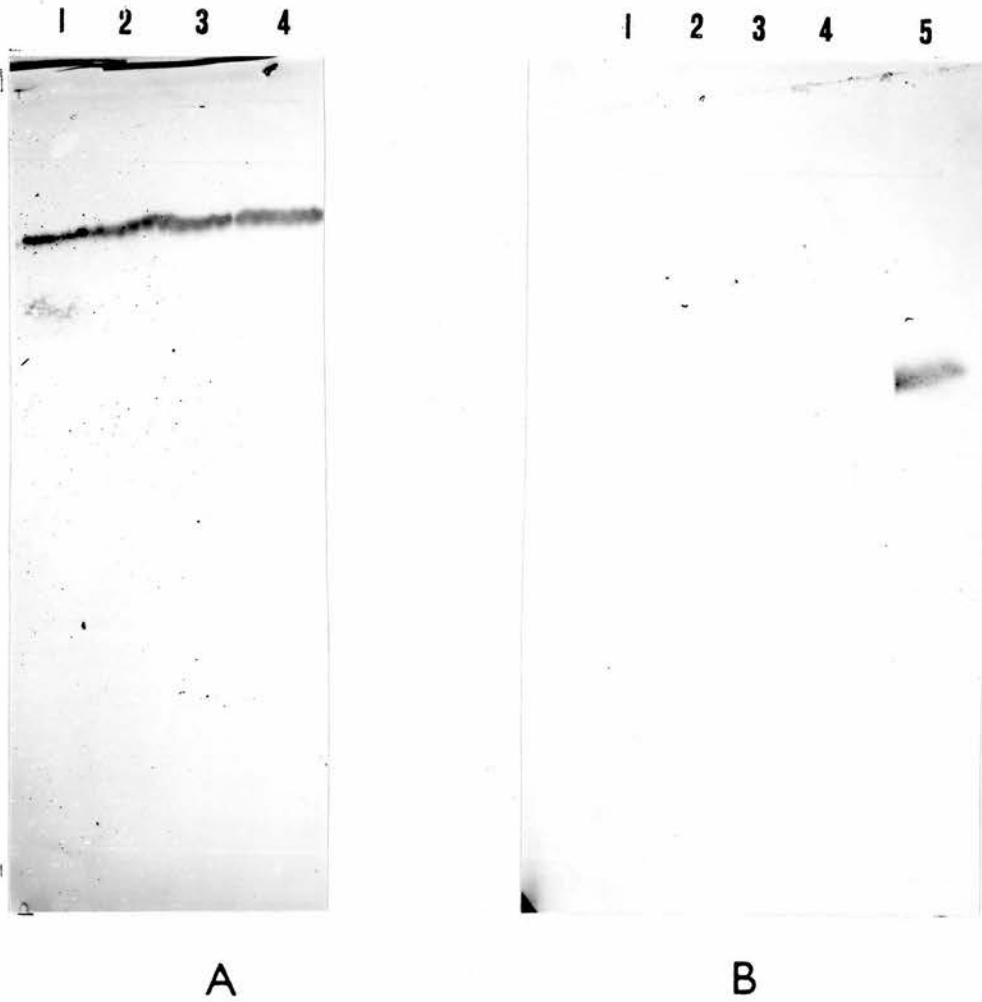


Figure 7.6 Replicas of (A) unreduced and (B) reduced chromaffin granule lysate proteins decorated with culture supernatant containing antibody A4. Track 1, 50 μ g of lysate protein; track 2, 100 μ g of protein; track 3, 150 μ g of protein; track 4, 200 μ g of protein. Track 5, chromaffin granule membranes (150 μ g).

one-dimensional gels.

In summary, monoclonal antibody A4 recognized both soluble and membrane DBH but chromogranin A prevented A4 from binding to DBH in the lysate in the presence of 2-mercaptoethanol. The monoclonal antiserum did not recognize deglycosylated DBH (see section 7.6 for discussion).

7.3 Monoclonal antibody ASV48

Monoclonal antibody ASV48 (gift of Dr. J. L. Bixby, University of California) was produced by immunizing Balb/c mice with synaptic vesicles from rat brain. Antibody ASV48 recognizes a protein (p65; Mr 65000), which is exposed on the outer surface of rat brain synaptic vesicles (Matthew et al., 1981). Antigenic components of the protein are widely distributed in neuronal and neural secretory tissue in sharks, amphibia, birds and mammals. Glycoprotein H from chromaffin granules migrates with an apparent Mr of 64000 in polyacrylamide gels under reducing conditions (Gavine et al., 1984) so experiments were carried out to investigate whether antibody ASV48 recognizes GpH.

Figure 7.7A shows a replica of a one-dimensional gel of chromaffin granule membrane proteins decorated with an ascites fluid containing ASV48 (diluted 100-fold in serum buffer). The antibody recognized a protein of apparent Mr 65000 and this could be GpH because it migrates similarly, as a narrow band. A replica of a two-dimensional gel of chromaffin granule membranes dec-

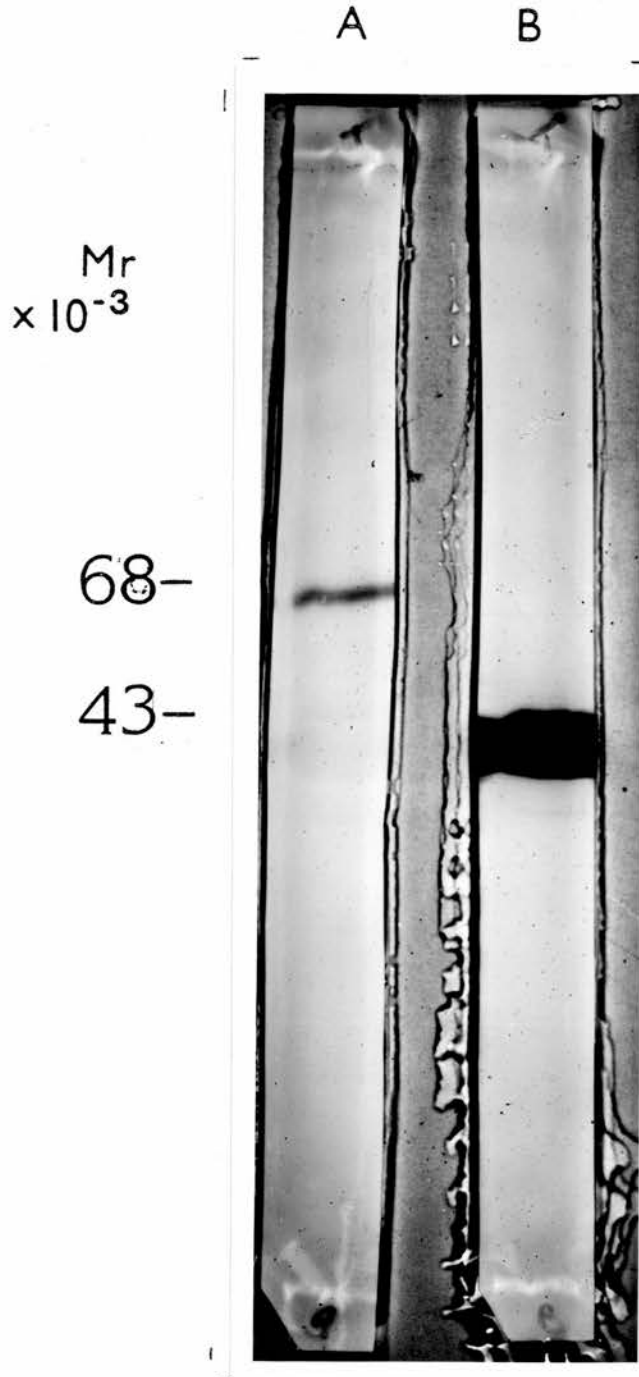


Figure 7.7 One-dimensional gels (6-15%) of chromaffin granule membranes (150 μg per track). Replicas were decorated with : (A) ascites fluid containing ASV48 (diluted 100-fold in serum buffer); (B) ascites fluid containing SY38 (diluted 100-fold in serum buffer).

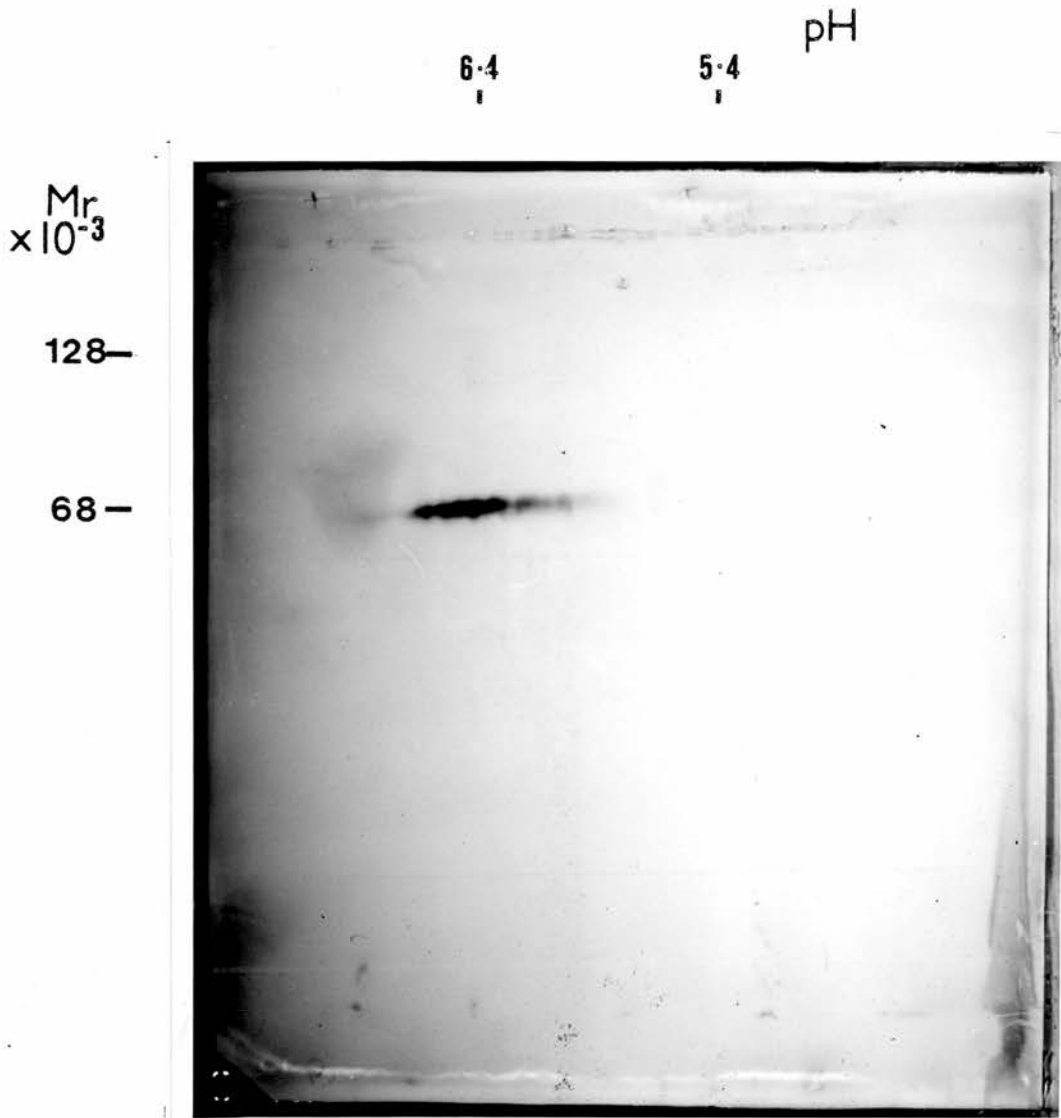


Figure 7.8 Replica of a two-dimensional gel of chromaffin granule membranes (300 μ g) decorated with ascites fluid containing ASV48.

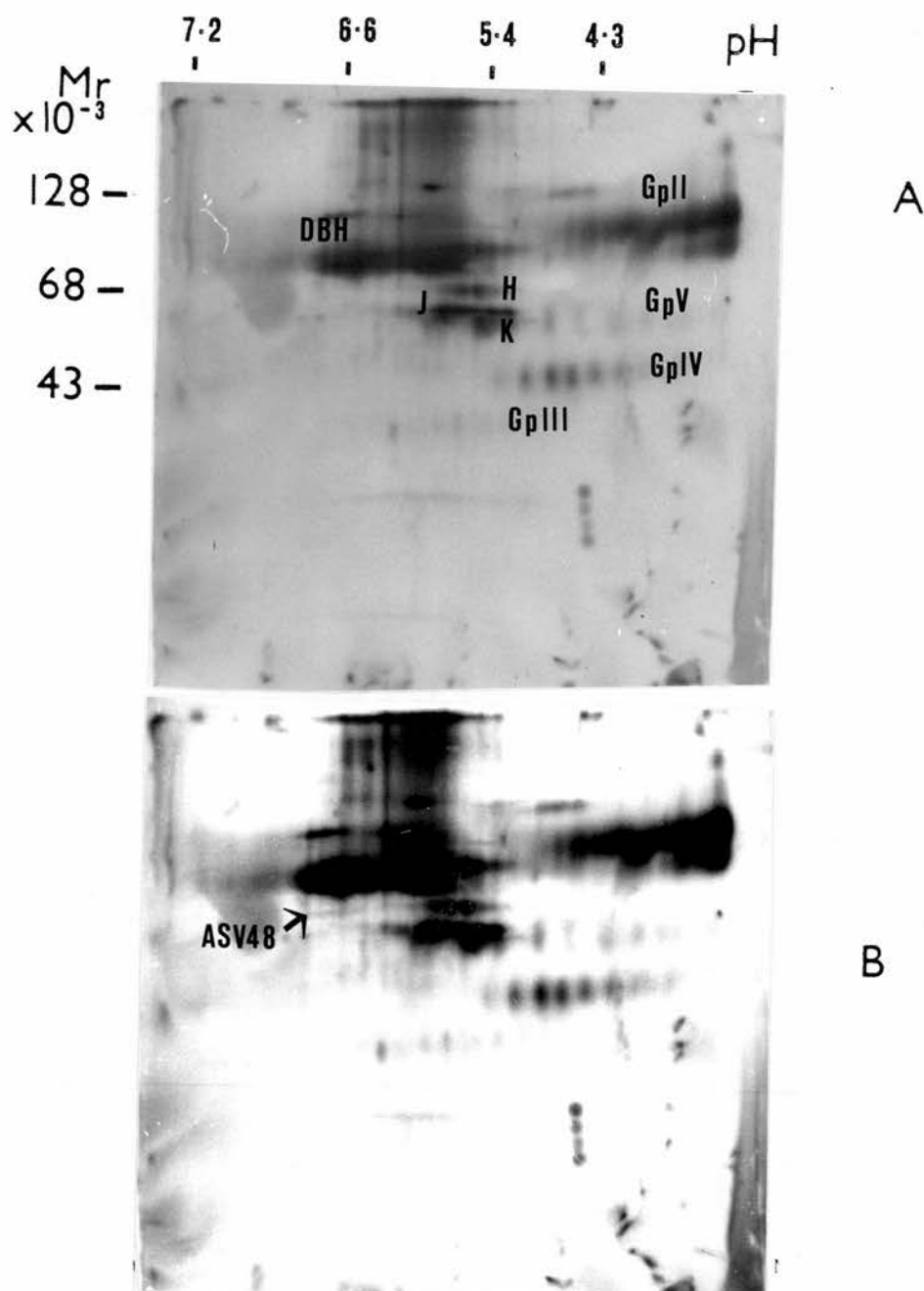


Figure 7.9 Replica of a two-dimensional gel of chromaffin granule membranes (300 μ g). (A) Autoradiograph of a replica decorated with ascites fluid containing ASV48 (diluted 100-fold) followed by 125 I-labelled concanavalin A. (B) Autoradiograph in (A) superimposed on the replica decorated with ASV48 shown in fig. 7.8.

Abbreviations: DBH, dopamine β -hydroxylase; GpII, glycoprotein II; GpIII, glycoprotein III; GpIV, glycoprotein V.

orated with ASV48 is illustrated in figure 7.8. ASV48 recognized a protein of Mr 65000, pI 6.5-6.8, but GpH focusses at the more acidic pH of 5.5. After photography, the replica in fig. 7.8 was incubated with BSA buffer, washed and then incubated with ^{125}I -labelled Con A and autoradiographed (see fig. 7.9A). The autoradiograph (shown in figure 7.9A) was superimposed on the replica (illustrated in figure 7.8) and the result is presented in figure 7.9B. ASV48 recognized a novel protein whose pI is more basic than that of GpH, and which does not bind Con A. However, WGA labelled a glycoprotein with a similar apparent Mr and pI to that of the antigen recognized by ASV48 (see fig. 7.11).

7.4 Monoclonal antibody SY38

Monoclonal antibody SY38 was produced by immunizing Balb/c mice with coated vesicles from bovine brain (Wiedenmann and Franke, 1985). Synaptophysin, an acidic glycoprotein (Mr 38000, pI 4.8), has been identified as a specific component of the membrane of presynaptic vesicles, using the monoclonal antibody SY38. Synaptophysin is also found in the adrenal medulla and in diverse vertebrate species. In its general properties synaptophysin appeared to be quite similar to Gplll.

A replica of a one-dimensional gel of chromaffin granule membranes was decorated with an ascites fluid containing SY38 (gift of Dr. B. Wiedenmann) to investigate whether SY38 labels Gplll (Mr 37000-43000, pI 4.6-5.3). Figure 7.7B shows that SY38 reacted with a protein (apparent Mr 40000) that migrated as a broad and poorly

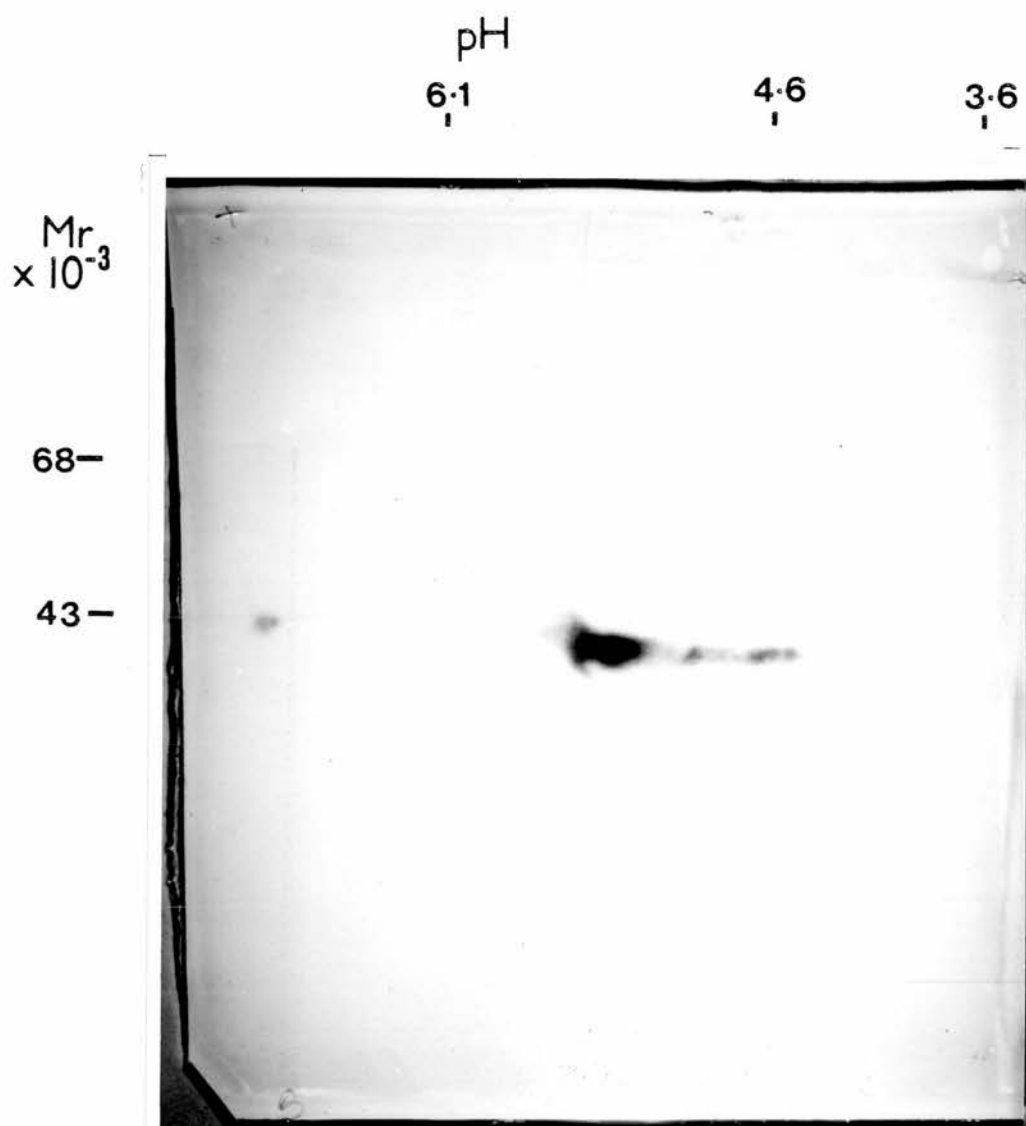


Figure 7.10 Replica of a two-dimensional gel of chromaffin granule membranes (300 μ g) decorated with an ascites fluid containing SY38 (diluted 100-fold).

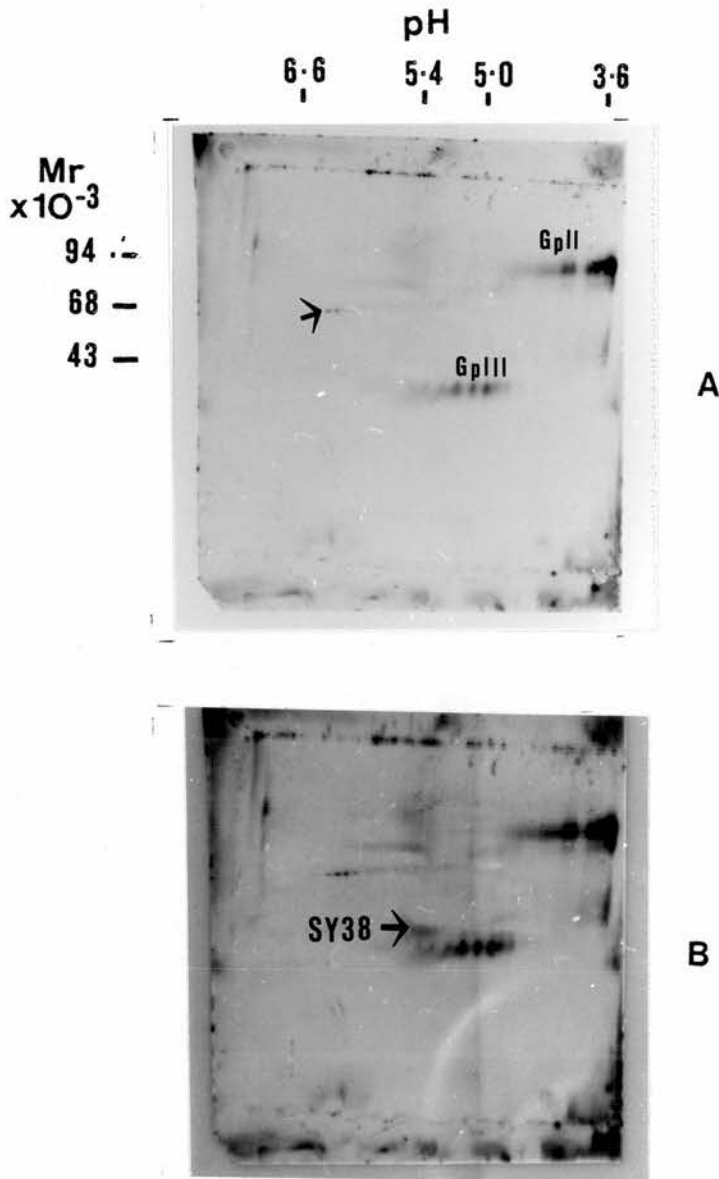


Figure 7.11 Replica of a two-dimensional gel of chromaffin granule membranes (300 μg). (A) Autoradiograph of a replica decorated with ascites fluid containing SY38 (diluted 100-fold) followed by ^{125}I -labelled WGA. (B) Autoradiograph in (A) superimposed on the replica decorated with SY38 shown in fig 7.10. The glycoprotein indicated by the arrow in (A) may be the antigen recognized by ASV48 (section 7.3). Abbreviations: GpII, glycoprotein 11; GpIII, glycoprotein 111.

focussed band, similar to that of Gplll. A replica of a two-dimensional gel of membranes decorated with SY38 (fig. 7.10) revealed a poorly focussed spot (pI 4.8 - 5.4) which was unlike the ladder of spots corresponding to Gplll on a two-dimensional gel. After photography, the replica illustrated in figure 7.10 was decorated with ^{125}I -labelled WGA to identify Gplll (see table 3.1) and autoradiographed. The autoradiograph (see fig. 7.11A) was superimposed on the replica (shown in fig. 7.10) and the result is presented in figure 7.11B. SY38 labelled a novel protein which did not bind ^{125}I -labelled WGA (or Con A) and which had a more basic pI and higher apparent Mr than Gplll.

7.5 Discussion

Monoclonal antibody A4 was produced which recognized both soluble and membrane DBH. The mouse was immunized with chromaffin granule membranes for monoclonal antibody production, suggesting a strong selection for the most immunogenic component. Nolan et al., 1985 also report production of a monoclonal antibody that reacts with both forms of DBH. Chromogranin A prevented A4 from binding on a replica of a one-dimensional gel of lysate proteins reduced with 2-mercaptoethanol. Antibody A4 did not react with deglycosylated DBH and it is possible that it recognizes an antigenic determinant on the carbohydrate portion. In most cases, the antibody recognized a single band (apparent Mr 74000) on a replica of a one-dimensional gel, whereas staining with Coomassie Blue, lectins or polyclonal antisera reveal three bands (see

chapters 3 and 4). In one case (track 3, fig. 7.5), the monoclonal antiserum revealed three bands when an unusually heavy loading of purified soluble DBH was used; this may indicate some structural or biosynthetic relationship between the major and minor bands of soluble DBH.

On a replica of a two-dimensional gel of membranes, A4 reacted with the middle spot of membrane DBH (component F of Gavine et al., 1984), whereas the polyclonal antiserum (see chapter 4) labelled all three components (E, F and G). Saban et al. (1983) and Saxena and Fleming (1983) report that membrane DBH is composed of two types of subunit which differ in apparent Mr. My results indicated that F differs from components E and G, and F might be common to both membrane and soluble DBH since A4 recognized both forms. Nevertheless, it is difficult to reconcile the appearance of three spots on a two-dimensional gel with a structure composed of two types of subunits. In fact, an $\alpha_2\beta$ structure for the membrane-bound form of the enzyme would not be inconsistent with the data in the literature.

There are similarities between calcium-dependent exocytosis in neuronal, endocrine and exocrine cells suggesting that some features of the biochemical mechanism of this process might be shared. If the shared proteins are highly conserved, all cells that have the regulated secretory pathway should contain common antigenic determinants. It was therefore interesting

that monoclonal antibodies SY38 and ASV48, which react with synaptophysin and p65 from synaptic vesicles, recognized two novel proteins on replicas of chromaffin granule membranes. The protein recognized by ASV48 was not labelled by Con A, but it did apparently bind WGA and in contrast, the antigen recognized by SY38 was not labelled by WGA. A monoclonal antibody was raised against SV2, a transmembrane glycoprotein (apparent Mr 100000) from cholinergic synaptic vesicles found in the electric organ of the elasmobranch Discopyge ommata (Buckley and Kelly, 1985). This antibody recognizes an identical antigen in several types of endocrine tissues in the rat, including the adrenal medulla, pituitary and pancreas. However, antibodies SY38, ASV48 and SV2 do not react with exocrine cells (Matthew et al., 1981; Buckley and Kelly, 1985; Wiedenmann and Franke, 1985). The monoclonal antibody to SV2 also recognized a protein, which was not identical with Gp11 (which has a similar Mr), after transfer of chromaffin granule membrane proteins to cellulose nitrate (J. G. Pryde personal communication).

Chromogranin A has been found in several endocrine tissues (O'Connor et al., 1983; Cohn et al., 1982) and in brain (Somogyi et al., 1983). Chromogranin C is immunologically identical with secretogranin 11 from pituitary (Rosa and Zanini, 1983; Fischer-Colbrie et al., 1986), and immunologically identical antigens to cytochrome b-561 (Duong et al., 1984) and Gp111 (Fischer-Colbrie

et al., 1984) are also found in the pituitary.

The discovery of antigens in several endocrine and neuronal tissues from different species suggests a conserved function for these proteins. SV2, p65 and synaptophysin were not found in exocrine cells and so there may be proteins which control biogenesis and exocytosis common to endocrine and neuronal tissues that are not found in exocrine tissues.

CHAPTER EIGHT
DISCUSSION

Glycoprotein J (apparent Mr 57000), glycoprotein K (apparent Mr 53000), Gp111 and DBH were solubilized from chromaffin granule membranes by 1% w/v octaethylene-glycol dodecyl ether and purified by affinity chromatography on LCL-agarose followed by electroelution from SDS polyacrylamide slab gels. Lubrol PX, Lubrol WX and Tween 20 failed to solubilize GpJ and GpK from the membrane suggesting that these are integral membrane proteins. The electroeluted proteins were judged to be pure by electrophoresis; the gels were stained with Coomassie Blue or replicas of the gels were decorated with Con A. Electroeluted glycoproteins were injected into guinea pigs and rabbits, and the polyclonal antisera produced were used to characterize the glycoproteins.

GpJ and GpK were isolated from a highly purified fraction of chromaffin granules but this does not prove that they are specific components of these organelles. However, immunological analyses of their distribution in membranes separated by sucrose density gradient centrifugation clearly showed that GpJ and GpK are specifically confined to chromaffin granules. They are apparently absent from other membranes in the adrenal medulla, e.g. mitochondrial or microsomal membranes. In addition, antiserum against glycoproteins J and K reacted with the cytoplasm of chromaffin cells as demonstrated by immunofluorescence microscopy. Thus in adrenal medulla GpJ and GpK, like membrane DBH,

membrane Gplll and cytochrome b-561 (Fischer-Colbrie et al., 1984; Winkler, 1976), represent specific markers for chromaffin granule membranes. Glycoproteins J and K were not found in the granule matrix in contrast to DBH and Gplll.

There have been several reports (Huber et al., 1979; Abbs and Phillips, 1980; Fischer-Colbrie et al., 1984) that membrane DBH and Gplll face the content of chromaffin granules. The proteolysis experiments in chapter 6 confirm these findings, there being no evidence for the exposure of Gplll and DBH on the exterior of these organelles. The WGA receptors on the cytoplasmic surface of chromaffin granules reported by Meyer and Burger (1976) thus cannot be Gplll molecules, and, since Gplll is a major WGA - binding species of the membrane, it seems likely that these workers' granule preparation was contaminated by membrane fragments. Glycoproteins J and K had trypsin-sensitive sites exposed on both sides of the granule membrane, suggesting that they are transmembrane glycoproteins. The location of the antigenic sites of glycoproteins J and K could not be determined from the proteolysis experiments, but complement fixation using intact granules and broken membranes is a good method for locating antigenic determinants. This was unsuccessful, however, because the anti-glycoproteins J and K serum had anti-complement activity, and although this anti-serum could be used for decorating cellulose nitrate

replicas it was unsuitable for complement fixation because it labelled other polypeptides as well as glycoproteins J and K (see chapter 4). The carbohydrate portions of glycoproteins J and K bind Con A strongly and are likely to be exposed on the inner (matrix) surface of the granule membrane since intact granules are not labelled by Con A or galactose oxidase/ KB^3H_4 (Eagles et al., 1975; Huber et al., 1979; Abbs and Phillips, 1980).

Electroeluted GpJ and GpK were injected into separate guinea pigs in order to raise antibodies but both guinea pigs produced an antiserum which recognized both glycoproteins. This raises the question of whether GpJ and GpK are related proteins with common antigenic determinants. The organization of the glycoproteins in the membrane and their lectin binding properties (table 3.1) appear to be similar, although the band of GpJ was usually darker than that of GpK on ^{125}I -labelled Con A overlays (fig. 3.1 and 5.12). GpJ may contain a higher proportion of high-mannose oligosaccharides which bind Con A, than GpK. An antigen migrating to the same region of the gel as GpK was found together with Gplll in the pituitary (section 6.3). However, non-specific binding of the anti-glycoproteins J and K serum to other proteins on the replica (fig. 6.5) made it very difficult to identify glycoproteins J and K in fractions from bovine brain.

Glycoproteins J and K, and Gplll are major glycoproteins of the granule membrane and yet their biological role is quite unknown. No enzyme activity has been detected in Gplll preparations which were tested for trypsin-like and γ -glutamoyl transferase activities, and for the ability to catalyse ATP-ADP exchange (Fischer-Colbrie et al., 1984).

Glycoproteins J and K, membrane Gplll, membrane DBH, and cytochrome b-561 can serve as markers for the membranes of chromaffin granules and should prove useful for studying granule biogenesis and the biochemical mechanism of exocytosis.

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techniques have failed. One possible approach to separate the hexose and nucleoside transporters is by immunoaffinity chromatography. In this report, we describe the properties of two monoclonal antibodies raised against a partially purified preparation of human erythrocyte band 4.5 polypeptides.

A partially purified preparation of nucleoside transporter protein(s), prepared by the method of Jarvis and Young (1981), was used as the immunogen after removal of detergent by treatment with Biobeads. Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis confirmed that the preparation consisted largely (>95%) of band 4.5 polypeptides, and detergent-free material was shown to be capable of reversibly binding nitrobenzylthioinosine (NBMPR). BALB/c mice were injected intraperitoneally with the immunogen (30 µg) and boosted three times 13, 24 and 27 days after the initial injection. One day after the last injection, mice were killed and spleen cells were fused with myeloma P3/NSI/1-Ag4-1 cells (Köhler & Milstein, 1976; Galfré & Milstein, 1981). Culture supernatants from hybridoma clones were initially screened for antibodies against protein-depleted human erythrocyte membranes using an enzyme-linked immunosorbent assay system and those that were positive were then screened for antibodies that immunoprecipitated nucleoside or glucose transporter proteins (Fig. 1) or that inhibited nucleoside transport by human erythrocytes. Two immunoglobulin G (IgG)-producing hybridomas (64C7 and 65D4) were selected for further study and were subcloned twice. Antibodies used in this report were isolated from ascites fluid or from cell-culture supernatants and were further purified by ammonium sulphate precipitation and protein A-Sepharose chromatography.

A direct interaction between the antibodies and transporter proteins was demonstrated by immunoprecipitation of solubilized human erythrocyte membranes photo-labelled with either [³H]NBMPR or [³H]cytochalasin B, specific inhibitors of nucleoside and hexose transport, respectively (Fig. 1). Band 4.5 proteins are selectively labelled by both ligands (Carter-Su *et al.*, 1982; Wu *et al.*, 1983). Antibody 65D4 immunoprecipitated both [³H]NBMPR- and [³H]cytochalasin B-labelled membranes (see Fig. 1). Similar results were obtained with antibody 64C7 (data not shown). In contrast, polyclonal mouse IgG prepared from non-immunized animals did not immunoprecipitate radioactivity. Electrophoretic transfer of human erythrocyte membrane proteins to nitrocellulose confirmed that both antibodies reacted with band 4.5, although antibody 64C7 also reacted with proteins with an apparent *M_r* of 70 000–90 000. Control experiments with non-immune mouse IgG did not label any protein bands. Additional electrophoretic studies with trypsin-treated 'ghosts' demonstrated that antibody 65D4 reacted with a broad band (apparent *M_r* 40 000–26 000) whereas 64C7 reacted with a sharp 50 000 *M_r* band. Endoglycosidase F treatment of partially purified band 4.5 proteins resulted in conversion of the diffuse band to a sharp band at 46 000. Subsequent electrophoretic experiments demonstrated that antibodies 64C7 and 65D4 labelled a sharp peak corresponding to *M_r* 46 000 after endoglycosidase F treatment, suggesting that the antibodies were not reacting with carbohydrate residues.

In conclusion, antibodies 64C7 and 65D4 appear to react with both the nucleoside and glucose transporters although the site of recognition on the protein is probably different for the two antibodies. Preliminary experiments suggest that treatment of human erythrocytes with antibodies 64C7 and 65D4 did not inhibit (a) high-affinity binding of [³H]NBMPR or (b) zero-trans influx of [³H]-uridine or [³H]adenosine. Further studies are in progress to attempt to raise a monoclonal antibody specific for the nucleoside transporter.

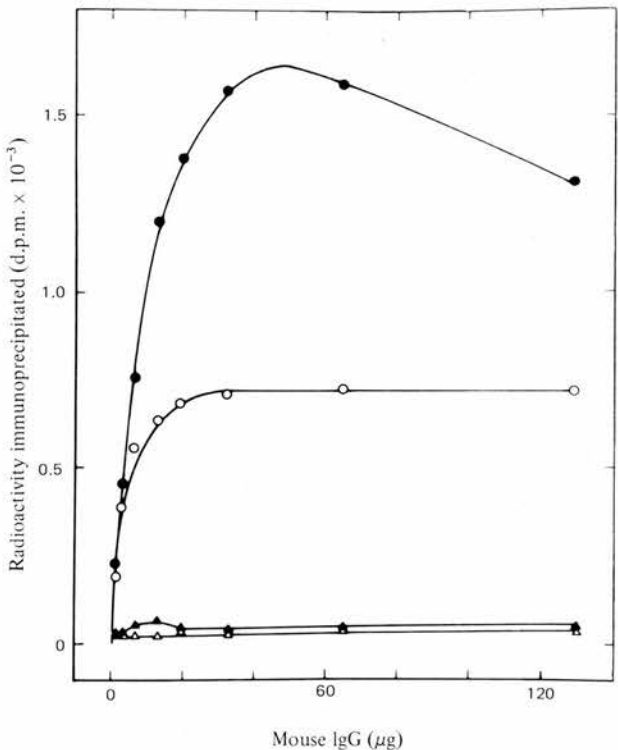


Fig. 1. Immunoprecipitation of the nucleoside and glucose transporters

'Protein-depleted' human erythrocyte membranes were equilibrated with either 60 nM-[³H]NBMPR, supplemented with dithiothreitol (final concentration 50 mM) or 2 µM-[³H]cytochalasin B, and exposed to high-intensity u.v. light from a 450 W mercury arc lamp (Wu *et al.*, 1983) for 45 s. Non-covalently bound radioactivity was removed by washing with 50 mM-Tris/HCl (pH 7.5) and the washed membrane pellets were solubilized with 1% Triton X-100. Non-solubilized membrane was removed by centrifugation (100 000 g, 1 h) and the solubilized membranes were incubated with purified 65D4 antibody (●, ○) or non-immune mouse IgG (▲, △) in a medium containing 1% Triton X-100, 100 mM-NaCl and 50 mM-Tris/HCl (pH 7.5). After 18 h, 100 µl of Pansorbin was added for 1 h at 4°C and the immunoprecipitates were washed twice by centrifugation at 10 000 g with 50 mM-Tris/HCl (pH 7.5) containing 0.1% Triton X-100. Radioactivity associated with the immunoprecipitates was determined by liquid scintillation counting ●, ▲, [³H]NBMPR-labelled membranes; ○, △ [³H]cytochalasin B-labelled membranes.

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Purification of chromaffin granule membrane glycoproteins by affinity chromatography on lectin columns

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Chromaffin granules are secretory granules, found in the adrenal medulla, which store and release catecholamines, adenine nucleotides and a number of proteins and peptides including the soluble form of dopamine β -hydroxylase (DBH, EC 1.14.17.1), chromogranins and enkephalins. The granule membrane contains approx. 40 proteins (Abbs & Phillips, 1980), the major components being DBH and cytochrome *b*₅₆₁ (see Fig. 1).

There have been several studies of chromaffin granule membrane glycoproteins. Huber *et al.* (1979) used periodic acid/Schiff stain on SDS/polyacrylamide gels and

Abbreviations used: DBH, dopamine β -hydroxylase; SDS, sodium dodecyl sulphate; LCL, *Lens culinaris* lectin; Con A, concanavalin A; Hepes, 4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid; C₁₂E₈, octaethyleneglycol dodecyl ether; WGA, wheat-germ agglutinin.

detected five glycosylated components. Cahill & Morris (1979) and Abbs & Phillips (1980) identified 12 glycoproteins on gels by using fluorescein-conjugated lectins. More recently a larger number have been identified after transfer to nitrocellulose sheets and decoration with radioiodinated lectins (Gavine *et al.*, 1984). We describe here the purification of three chromaffin granule membrane glycoproteins of unknown function, as well as membrane-bound DBH, by chromatography on lectin affinity columns.

Chromaffin granule membranes were isolated from fresh bovine adrenal glands (Apps & Schatz, 1979). *Lens culinaris* lectin (LCL)-agarose and concanavalin A (Con A)-agarose columns were pre-washed with 20 mM-Hepes/NaOH/0.5 M-NaCl/0.1% (w/v) octaethyleneglycol dodecyl ether (C₁₂E₈), pH 7.4, at a flow rate of 4 ml/h at 4°C. Fresh membranes (2 mg of protein/ml) were solubilized with 1% (w/v) C₁₂E₈, 20 mM-Hepes/NaOH, pH 7.4, and diluted 20-fold with 20 mM-Hepes/NaOH, pH 7.4. Diluted solubilized membrane proteins (150 ml) were applied to a LCL-agarose column at a flow rate of 4 ml/h at 4°C; membrane proteins not adsorbed by this column were immediately applied to a Con A-agarose column at the same flow rate. The columns were washed with 50 ml of 20 mM-Hepes/NaOH/0.5 M-NaCl/0.1% (w/v) C₁₂E₈, pH 7.4, at a flow rate of 4 ml/h and eluted 'batchwise' with 10% (w/v) α -methylmannoside in the same buffer. Effluent and eluate fractions from the columns were freeze-dried, redissolved in a minimum volume of distilled water and analysed by SDS/polyacrylamide-gel electrophoresis (Laemmli, 1970) in the presence of 2-mercaptoethanol.

Fig. 1 shows a Coomassie Blue-stained electrophoretic separation of chromaffin granule membrane proteins, and of proteins eluted from LCL-agarose and Con A-agarose. DBH (*M*_r 75 000), glycoprotein J (*M*_r 56 000), glycoprotein K (*M*_r 53 000) and glycoprotein R [glycoprotein III of Huber *et al.* (1979), *M*_r 37 000] were eluted from LCL-agarose. These glycoproteins were further purified by electroelution of one-dimensional electrophoretograms in SDS of the eluate from LCL-agarose. Fig. 1 shows a one-dimensional SDS gel of electroeluted DBH, stained with Coomassie Blue; similar results were obtained with the other glycoproteins.

Chromaffin granule membrane proteins were separated by one-dimensional electrophoresis, transferred to nitrocellulose sheets (Towbin *et al.*, 1979) and decorated with ¹²⁵I-Con A, ¹²⁵I-LCL and ¹²⁵I-wheat germ agglutinin (WGA). Autoradiographs of the nitrocellulose blots show that DBH is labelled by all three lectins, glycoproteins J and K by Con A only and glycoprotein R by WGA and LCL but not by Con A. After one-dimensional electrophoresis, the eluate from LCL-agarose, electroeluted glycoproteins J and K and electroeluted DBH were transferred to nitrocellulose, which was then decorated with Con A and horseradish peroxidase (Clegg, 1982). The nitrocellulose transfer showed that DBH and glycoproteins J and K are eluted from LCL-agarose and single bands of electroeluted glycoproteins J and K and electroeluted DBH were labelled by Con A.

This purification of the chromaffin granule membrane glycoproteins R, J, K and DBH by affinity chromatography on lectin columns has yielded proteins that are sufficiently pure for raising antibodies in guinea pigs.

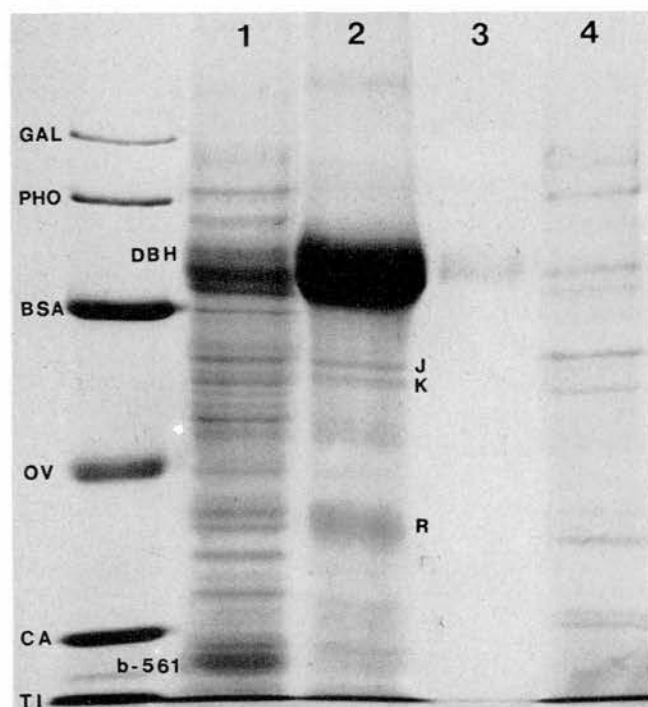


Fig. 1. One-dimensional SDS/polyacrylamide gel (acrylamide concentration 6–10%) stained with Coomassie Blue

Track 1: chromaffin granule membranes; track 2, eluate from LCL-agarose; track 3, electroeluted DBH; track 4, eluate from Con A-agarose. Standard proteins: β -galactosidase (GAL, *M*_r 128 000); phosphorylase (PHO, *M*_r 94 000); bovine serum albumin (BSA, *M*_r 68 000); ovalbumin (OV, *M*_r 43 000); carbonic anhydrase (CA, *M*_r 30 000); trypsin inhibitor (TI, *M*_r 21 500).

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Preparation and characterization of a K⁺-stimulated adenosine triphosphatase from the midgut of *Manduca sexta*

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The midgut of *Manduca sexta*, a phytophagous lepidopteran larva, transports large quantities of potassium ion from the blood side to the lumen side. (Thomas & May, 1984). Physiological studies have revealed that the most likely mechanism for this transport is a K⁺-stimulated, or K⁺-dependent, adenosine triphosphatase, (K⁺-ATPase) (Cioffi & Wolfersberger, 1983). There exists some indirect evidence to support the presence of a K⁺-ATPase in this tissue, and a few attempts have been made to isolate membrane fractions carrying K⁺-ATPase activity. Deaton (1984) used differential centrifugation to harvest a light microsomal fraction from midgut homogenates, and detected a low level of K⁺-ATPase activity (V_{\max} = 150 nmol of ATP/mg of protein per h). Other groups have attempted to prepare similar subcellular fractions by density gradient centrifugation but apparently without success (Wolfersberger, 1984). In an accompanying paper we describe the use of isopycnic sucrose density gradient centrifugation in a zonal rotor to prepare a fraction containing substantial amounts of K⁺-ATPase activity. (Keighley *et al.*, 1985). We describe here the results of experiments done to determine some of the characteristics of this enzyme, including its substrate specificity and its sensitivity to various inhibitors.

A membrane fraction enriched in K⁺-stimulated ATPase was prepared from *M. sexta* midgut homogenates as previously described (Keighley *et al.*, 1985). ATPase was determined using the following modification of the method described by Peacock (1981).

Fractions from a sucrose density gradient were preincubated in a buffer system (50 mM-imidazole/HCl, pH 7.2, containing either: no cations; 5 mM-Mg²⁺; 5 mM-Mg²⁺ and 25 mM-K⁺ or 5 mM-Mg²⁺, 25 mM-K⁺ and 25 mM-Na⁺; ions were all added as their chloride salts). The reaction was started by the addition of Tris/ATP to a final concentration of 3 mM. The reaction was allowed to proceed for 1 h at 22°C then stopped by the addition of phosphate-detection reagent containing 0.5% (w/v) sodium dodecyl sulphate, pH 4.0 (Atkinson, 1972). K⁺ stimulation was assumed to occur when the presence of K⁺ increased the rate of hydrolysis of ATP above that occurring in the absence of K⁺. The optimal pH of the enzyme was determined using a number of Goods buffers at 0.5 pH intervals over the pH range 5–12. Substrate specificity was determined by substituting the ATP in the assay by either TP, AMP, ADP or *p*-nitrophenyl phosphate (p-NPP). The effects of different concentrations of various inhibitors was determined using incubation mixtures containing 3 mM-ATP, 5 mM-Mg²⁺ and 0 or 25 mM-K⁺.

Table 1. Effects of inhibitors on K⁺-ATPase activity

Inhibitor	Concentration (M)	K ⁺ -ATPase activity (% of control)
Ouabain	10 ⁻⁵	79
Vanadium	10 ⁻⁴	104
AC24055	10 ⁻⁸	33
Valinomycin	10 ⁻⁴	105
Trimethyl tin chloride	10 ⁻⁴	95
Concanavalin A	10 ⁻⁵	25
Ethacrynic acid	10 ⁻⁴	76
Ethylmaleimide	10 ⁻⁴	43
Oligomycin	10 ⁻⁶	94
9-Amino acridine	10 ⁻⁸	18

The K⁺-ATPase was shown to have a K_m for ATP of 2.66 μM and a pH optimum of pH 7.2. K⁺ stimulation was found to be optimal at a K⁺ concentration of 25 mM where, in a typical preparation of the enzyme, a 2.5-fold stimulation of ATPase activity was observed. The addition of up to 200 mM-Na⁺ gave no further stimulation of activity. The maximum velocity of the enzyme determined in these experiments was 8.5 μmol of ATP hydrolysed/mg of protein per h.

The membrane fraction was also shown to have enzyme activity able to hydrolyse ITP and AMP. AMP is hydrolysed much more slowly than ATP, and neither AMP nor ITP hydrolysis is stimulated by K⁺. ADP is not hydrolysed, nor is p-NPP, at the pH optimum of the ATPase.

Table 1 summarizes the effects of a number of inhibitors on the K⁺-ATPase. Measurement of the effects of inhibitors in the presence and absence of K⁺ has enabled us to decide whether the inhibitor acts at the level of the K⁺-ATPase or at the level of the ATPase activity which is not stimulated by K⁺. Of particular interest is the observation that the American Cyanamid insecticide AC24055, at a concentration of 10⁻⁸ M, completely blocked the stimulation of the ATPase by K⁺. This observation compares with the findings of Thomas & May (1984), who showed that this compound completely and irreversibly blocks K⁺ transport when applied at a concentration of 10⁻⁴ M to the haemolymph side of tissue mounted *in vitro*.

In summary the results of this investigation are consistent with the suggestion that the K⁺-ATPase activity identified in a membrane fraction isolated from the midgut of *M. sexta* represents the K⁺ pump previously described in this tissue.

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Abbreviation used: p-NPP, *p*-nitrophenyl phosphate.

Putative precursors of rat liver cytosolic and mitochondrial fumarases

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Pairs of cytosolic and mitochondrial isoenzymes are generally encoded at different genetic loci and the protein products of these loci may be structurally related (e.g. aspartate aminotransferases) or unrelated (e.g. superoxide dismutases); for a review of such structural relationships see Doonan *et al.* (1984a). The fumarases are different. Edwards & Hopkinson (1979) provided genetic evidence that the isoenzymes of fumarase, although electrophoretically distinct, are encoded at the same locus. Subsequently O'Hare & Doonan (1984, 1985) purified the fumarases from pig liver and showed that they have nearly identical structures differing from one another only in the *N*-terminal region. Similar observations have been reported for the isoenzymes from rat liver (Kobayashi & Tuboi, 1983).

These results raise interesting questions about the biosynthesis of the isoenzymes. One possibility is that the cytosolic fumarase is the primary translation product and that the mitochondrial form is generated from it by removal of a short section from the *N*-terminus during import into the mitochondria (Doonan *et al.*, 1984b); the piece removed would have to be small since the isoenzymes are not resolved by electrophoresis in the presence of sodium dodecyl sulphate (O'Hare & Doonan, 1985).

To test this possibility we have studied the synthesis of fumarases *in vitro*. Total polysomes were isolated from rat liver as described by Kraus & Rosenberg (1982) and the polysomes translated in a message-dependent rabbit reticulocyte lysate supplied by The Radiochemical Centre, Amersham, U.K. The lysate was used exactly as described by the suppliers; translation mixtures contained 5 μ Ci of [³⁵S]methionine and, where appropriate, 1.5 *A*₂₆₀ units of polysomes. Protein samples were analysed by electrophoresis in the presence of sodium dodecyl sulphate as described by Laemmli (1970) and the electrophoretograms stained for protein before fluorography.

A typical experiment is shown in Fig. 1. Track 3 contained translation products immunoselected by an anti-fumarase antiserum active against both the cytosolic and mitochondrial isoenzymes; the immune complexes were isolated by absorption on to fixed *Staphylococcus aureus* cells. It can be seen that two radioactive proteins were immunoselected and moreover that both of these migrated more slowly than native pig heart fumarase, the position of which is shown in tracks 1 and 6. Neither of the components in track 3 originated from the lysate. Unprogrammed lysate produced one major band (track 4) but this did not combine with the anti-fumarase antiserum (track 5).

In a separate experiment (results not shown) rat liver fumarase immunoprecipitated from a total tissue homogenate was run on the gel along with a set of standard proteins as molecular weight markers. The subunit *M_r* values of native fumarase and of the two putative pre-fumarases were estimated as 47 000, 51 800 and 55 000 respectively. In the same experiment it was shown that addition of rat liver homogenate containing about 2.6 μ g of fumarases to the cell-free system before addition of antiserum effectively prevented precipitation of the putative precursors; this provided confirmation that both of the products were pre-fumarases.

These results indicate that two precursors of fumarase are synthesized in cell-free systems with *M_r* values about 4000 and 7000 greater than that of the native form. It is possible, but unlikely, that the smaller product originated from the larger one by non-specific proteolysis. It is

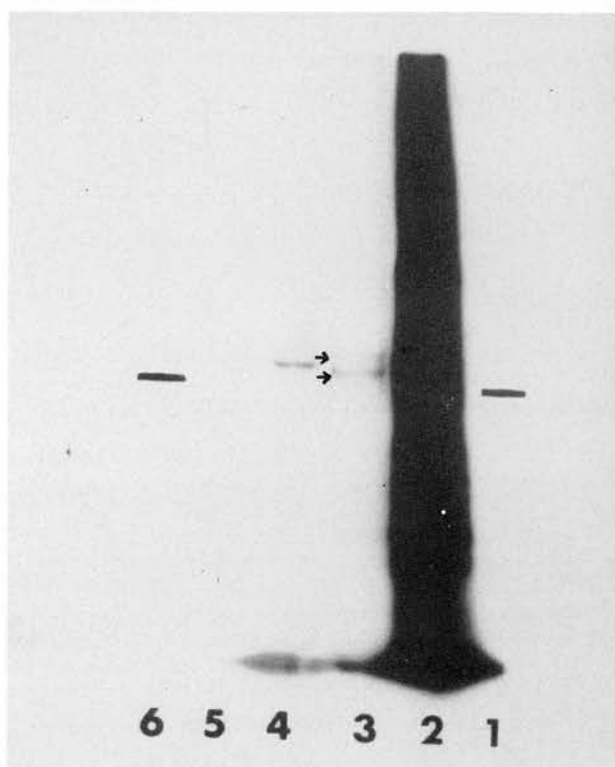


Fig. 1. Analysis by gel electrophoresis of translation products from total liver mRNA

Lanes contained the following: 2, total translation products; 3, as in 2 after immunoselection with antifumarase antiserum; 4, translation products with no added mRNA; 5, as in 4 after immunoselection with anti-fumarase antiserum. The fluorogram was overlaid on the gel stained for protein and the positions of native fumarase marked in lanes 1 and 6.

more likely, however, that both are genuine precursors, one of the cytosolic isoenzyme and the other of the mitochondrial form, and that they are translated from two different mRNAs. Given that the fumarases are coded at the same locus (see above) then the two mRNAs would have to originate from the same gene. There are precedents for this for example in the case of cytosolic and secreted forms of invertase in yeast (Perlman *et al.*, 1982). The two mRNA might arise by differential splicing of a primary transcript or by initiation of transcription at two distinct promoters. Experiments are in progress to distinguish between these possibilities in the case of fumarases.

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